MORPHOLOGICAL AND HISTOCHEMICAL STUDIES ON THE EFFECTS OF L-AZETIDINE-2-CARBOXYLIC ACID ON SKELETOGENESIS IN DEVELOPING CHICK EMBRYOS

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ABSTRACT

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Morphological and Histochemical Studies on the Effects of L-Azetidine-2-Carboxylic Acid on Skeletogenesis in Developing Chick Embryos

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Fertile eggs of White Leghorn chickens were injected with 0.6 cc of 1 mg/ml solution of L-azetidine-2-carboxylic acid (LACA) through the air sac. The experimental embryos were divided into 3 groups. Group I received one injection at pre-incubation; Group II 2 injections at pre-incubation and 48 hr; Group III 3 injections at pre-incubation, 48 hr, and 96 hr. The embryos for the gross morphological studies were sacrificed on days 7, 9, and 11 and analyzed on the basis of calcium deposition in the skeleton. An orange-red dye lake was observed in the metatarsus, tibiofibula, ribs, and the anterior regions of the radioulna in day 7 of both control and experimental Group I embryos. The orange-red dye lake was not observed in the phalanges, metacarpalia, and cranio-vertebral regions at this age in both groups. In the experimental Groups II and III, a faint

orange-red color was observed in the regions of the metatarsus, tibiofibula, femur, and radioulna; dye formation was not observed in any other regions.

In 9-day-old embryos, the dye formation was observed to be well advanced in the metatarsus, tibiofibula, ribs, and radioulna regions in both the control and the experimental Group I. The formation of the dye in experimental Groups II and III in 9-day embryos was comparable to that of control and experimental Group I of 7-day-old embryos. Although the formation of the dye was observed in all of the skeletal regions of the control, experimental Groups I, II, and III of 11-day-old embryos, the formation in the phalanges and the metacarpalia was less in Groups II and III.

Microscopic observations showed that in both the control and experimental Group I most of the matrix within the cartilage is basophilic, with few areas observed as acidophilic. But the perichondral regions or the peripheral regions of the cartilage were observed to be acidophilic. These indicated that sulfated and nonsulfated acid mucopolysaccharides were found within the cartilage, whereas the neutral mucopolysaccharides were seen within the perichondral or the peripheral regions of the cartilage. Within the cartilage of experimental Groups II and III, both matrix and the perichondral regions were observed to be acidophilic, with some of the matrix regions showing basophilia. This

indicated that both nonsulfated and sulfated acid mucopolysaccharides in these groups were suppressed, whereas neutral mucopolysaccharides were not.

Observations on the slides treated in diamine solutions showed that in the controls and the experimental Group I, the matrix was intensively stained with the diamine solutions, with few areas within the matrix showing Alcian blue staining. The presence of sulfated acid mucopolysaccharides was indicated by the diamine stain and nonsulfated acid mucopolysaccharides by Alcian blue stain. In experimental Groups II and III, the matrix was observed intensively stained with Alcian blue and mildly stained with diamine, indicating the presence of nonsulfated acid mucopolysaccharides is more than sulfated acid mucopolysaccharides. This suggests that LACA acts in some ways to inhibit the synthesis of sulfated acid mucopolysaccharides.

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CHAPTER I

INTRODUCTION

Certain plant families such as Liliaceae, Leguminosae (Fabaceae), etc. contain varieties of species with important economic value. Several are used as sources of cardiac clycosides, diuretics (Lewis, 1977); diaphoretics, emetics, antispasmodics, emmenagogues, expectorants, analgesics, anthelmintics (Krochman et al., 1971). The species that concern us here are Convallaria majalis (Lily-of-the-Valley), Polygonatum multiflorum, and DeLonix regia. These plants are widely cultivated and they contain many cardiac glycosides, of which the Convallaria majalis is the most significant. Some investigators indicated that poisoning has not been observed among these species, but cautioned that they are potentially dangerous due to their constituents and wide availability (Lewis, 1977). Others, however, reported that children have been fatally poisoned by drinking water from vases containing the flowers of digitalis-containing plants such as Convallaria majalis (Lampe, 1974).

A compound, L-azetidine-2-carboxylic acid (LACA), an analog of proline, was first isolated from Convallaria majalis by Fowden (1956) and from DeLonix regia (Sung and Fowden, 1969). It is a new cyclic imino acid that occurs naturally in many plant seeds, leaves, roots, and rhizomes, especially

among the families of Liliaceae, Agavoceae, and Leguminosae (Fabaceae), and LACA has the following derivatives: 5-dehydro-L-pipecolic acid and 4-hydroxy-L-proline.

Although representatives of these families are used as medicinal plants, the LACA isolated from them has not been reported to be used medicinally; however, it is used widely in laboratory experiments throughout the world. Among many investigators who reported its effects on plants and animals were Fowden (1963), who observed LACA to be a growth inhibitor in both E. coli and seedlings of the mung bean (Phaseolus aureus). It has been demonstrated by other investigators to inhibit the synthesis of collagen in a developing chick embryo (Lane et al., 1971a). Other effects of LACA that have been reported include failure of collagen excretion into the primary stroma of the cornea, fragility of bone in developing chick embryos (Lane et al., 1971b), and a failure of cells to excrete collagen (Takeuchi et al., 1969).

The collagen that is secreted by the Golgi apparatus is in the form of tropocollagen which complexes to produce fibrils, fibers, connective tissue such as cartilage, bone, tendon, etc. But after injection of the developing chick embryo with LACA, collagen failed to be secreted (Fowden, 1963; Lane et al., 1971a; Coulombre and Coulombre, 1972). Hepatic fibrosis, which is characterized by excessive deposition of newly synthesized liver collagen, has been studied in detail in rats, and LACA has been reported to effectively

inhibit the synthesis of liver collagen in these animals (Rifas and Selfter, 1977; Rojkind, 1973). Collagen synthesized in the presence of LACA is deficient in hydroxyproline, hydroxylysine, and glycosylated hydroxylysine (Takeuchi et al., 1969).

The present investigation, therefore, had as its objective to study the effects of LACA on skeletogenesis including a detailed examination of the effect of this proline analog on the production of glycosaminoglycans or acid mucopolysaccharides and calcification in developing chick embryos. Although the observations reported are not conclusive evidence of the effects of LACA on skeletogenesis in the chick embryo, they do, however, support the fact that LACA effectively disrupts skeletogenesis and calcification.

CHAPTER II

REVIEW OF LITERATURE

Natural plant drug materials are still economically significant in many parts of the world, in spite of increases in the production of synthetic drugs. A 1962 survey of new prescriptions written in the United States indicated that 25% were for drugs from natural plant products (Krochmal, 1971), and although there is great emphasis on the modern medicine, the herbalists are still reported to be practicing all over the world (Lewis, 1977). Many of these plants are poisonous; for instance, colchicine and other alkaloids are found throughout these plants, especially among the family Liliaceae (lily), and the poisoning has resulted in many deaths (Lewis, 1977). According to Lampe (1974), children were reported to have been fatally poisoned by drinking water from vases containing the flowers of digitalis-containing plants such as Convallaria majalis (Lily-of-the-Valley), Digitalis purpurea (foxglove), and Nerium oleander (oleander).

Convallaria majalis, Polygonatum multiflorum (Solomon's Seal), and DeLonix regia are species of plants belonging to the families Liliaceae and Leguminosae (Fabaceae), respectively. A compound, L-azetidine-2-carboxylic acid (LACA), was isolated from species of these plants (Fowden, 1956; Sung and Fowden, 1969; Grant et al., 1975). After the

isolation of L-azetidine-2-carboxylic acid, Fowden (1963) reported that this chemical was a growth inhibitor. It inhibited growth in E. coli and the seedlings of Phaseolus aureus (mung bean). Baich and Smith (1968) also showed that LACA slowed the rate of growth in E. coli. But it has been shown also that the growth of wild type E. coli was not affected by LACA when it was added to the medium in low concentration, as long as proline was present; but increasing the amounts of LACA in the medium progressively inhibited the growth of wild type. In contrast, growth inhibition was not detected in cultures of the mutant when the LACA concentration was increased 100 times greater than proline (Grant et al., 1975). In another study by Fowden and Richmond (1963), LACA was demonstrated to compete for transport of proline and for aminoacylation of proline t-RNA (Papas and Mehler, 1970). These strongly suggested that LACA had an adverse effect on the chemical system of the living organism.

Cells of the unicellular alga, Cyanidium caldarium, were inhibited from synthesizing chlorophyll-a and phycocyanin, but the inhibitory effect of the homolog on pigment synthesis in algal cells was partially reversed during subsequent incubation with proline (Troxler and Brown, 1974). Using the xylem of excised barley roots, Pitman et al. (1977) showed that at relatively low concentrations (50 μM) LACA was a potent inhibitor of potassium and chloride ion release to the xylem of excised roots of barley (Hordeum

vulgare) and intact plants. They demonstrated that LACA did not inhibit protein assembly, but the proteins formed are ineffective as enzymes. The uptake in roots was also inhibited but to a lesser degree.

In hydra, specialized cells known as nematoblasts synthesize the components of nematocysts, structures used to capture prey. Barzansky (1975) showed that at 24 hr. incubation newly-forming nematocysts showed irregularities in the outer capsule, and after 48 hr the arrangement of spines, thorns, and tube inside the capsule was abnormal. In an earlier study, Barzansky (1974) showed that LACA affected head regeneration in hydra.

Using the cultures of mouse limb buds for a period of 2 to 9 days, Aydelotte (1971) showed that LACA interferes with the normal development of cartilage. By increasing the concentration of LACA, he noted a delay of chondrogenesis and the formation of cartilage matrix which were both grossly and microscopically abnormal. Aydelotte and Kochhar (1972), after culturing chick limb buds for 2 to 14 days, observed that the growth of the entire limb bud was inhibited by LACA. They further noted a delay in chondrogenesis, and the cartilage which subsequently appeared became swollen, soft, and malformed. O'Dell (1966) reported that both the limb bone rudiments and the mandibular cartilage were bent and soft in the presence of LACA.

Coulombre and Coulombre (1971), having injected LACA

into the chorioallantoic vein of chick embryos, noted that silver-free patches appeared under the epithelium. Further studies showed that there were lesions produced in the primary corneal stroma. In another study Coulombre and Coulombre (1972) demonstrated that administration of LACA to growing chick embryos caused the Golgi apparatus not to stain with silver. However, when the animals were injected with both LACA and L-proline, no damage was seen when their corneas were examined.

Using other chemicals such as ^{3}H-proline, d-dipyridyl, or cis-hydroxy-proline, which are known to inhibit collagen production, Zamoraeva et al. (1970) observed that notochords of 12-72 hr chick embryos were rod-shaped and completely devoid of extracellular materials. The peripheral migration of notochordal cells, some assuming a fibroblastic appearance, was noted along a heavy general label over the notochordal cells. The evidence indicated that the notochordal ephithelium possessed a capacity for the production of extracellular connective tissue fibrils which are associated with a proline-containing notochordal secretion. This secretion can be markedly decreased by specific collagen inhibitors.

A strong inhibition of the incorporation of proline $-C^{14}$ into proteins of cartilage tissue by LACA was found to be increased with an increase in the period of its incubation with the tissue (Zamaraeva et al., 1969). The determination of the radioactivity of hydroxyproline showed that

collagen synthesis was depressed by LACA. But they also observed that LACA had a less marked inhibitory action on incorporation of glycine C^{14} into the proteins of cartilage tissues. After incubation of the tissue of the chick embryos with the analog for 5 to 7 days, they observed strong inhibition of the protein synthesis.

Inhibition in the growth of nine-day-old granulomas by LACA was demonstrated by Dancewicz and Altman (1967). The activity of the granuloma was severely prevented when the concentration of LACA was increased, and they concluded that LACA interfered with collagen synthesis. Cooper and Prockop (1967), after incubating connective tissues of 10-day chick embryos anaerobically, stated that the synthesis of collagen can be interrupted.

The abnormal polypeptides that were synthesized in the presence of LACA failed to be extruded into the extracellular matrix. Rather, they were retained intracellularly, as was demonstrated by Takeuchi et al. (1969). The collagen containing the analog was not extruded due to a decreased content of hydroxy-proline as well as a decreased content of hydroxylysine. The growth of E. coli was also demonstrated to be inhibited by LACA (Takeuchi and Prockop, 1969). They found that as a result of this inhibition the protocollagen that was synthesized differed kinetically from the normal protocollagen that was incubated with protocollagen proline hydroxylase. It was also noted that LACA incorporated into

an abnormal protocollagen which was subsequently converted to an abnormal collagen.

By addition of α-amino isobutyric acid, hydroxyproline, and LACA to the incubation medium, Finerman and Rosenbert (1967) were able to note a marked decrease in the rates of proline incorporation into protein and collagen. It was concluded from this experiment on rat tissue, that the alterations in intracellular proline concentrations or inhibiting uptake of proline into the intracellular pool, contributed to the change in the regulation of collagen synthesis.

Lane et al. (1971b) noted an increase of collagen degradation and fragility of the embryos when the proline analog azetidine-2-carboxylic acid was administered. They did this by treating chick embryos from day 8 to day 12 with 500 mg of LACA per day. When the collagen of the treated chick embryos was studied by electron microscopy, cross striations were seen in tendon fibrils. Analyzing the amino acid of the tendon revealed that the decrease in cross-striated fibrils was due to the decrease in collagen content of the tissue. When acid soluble collagen was isolated from the treated embryos, results showed this collagen to have essentially the same amino acid composition as normal collagen, the exception being that the collagen under study contained 4 residues of azetidine-2-carboxylic acid per 1000 residues of amino acid.

In other studies by Lane et al. (1971a), in which

1-5-day-old chick embryos were used, LACA arrested the accumulation of collagen in the embryos. The decrease in collagen content was accompanied by a marked increase in the fragility of the embryos. It was suggested, therefore, from the results that the decrease in collagen content of the embryos was due to the intracellular synthesis of a collagen which contained the analog and could not be extruded at a normal rate. It was also observed that after the cells had accumulated a large pool of analog-containing collagen, they reached a new steady state in which both the rate of collagen synthesis and extrusion were markedly decreased.

The cardiac jelly, the extracellular matrix of the developing chick heart, consists mainly of glycosaminoglycans. When embryos were cultured in the presence of either α, α-dipyridyl, or L-azetidine-2-carboxylic acid, the fusion of premyocardial mesoderm was not prevented by any of the agents. However, myocardial cytodifferentiation and normal cardiac morphogenesis were inhibited markedly (Johnson et al., 1973). Using the perinotochordal tissue of chick embryos, Frederickson (1974) observed that the notochordal boundary membrane-basal lamina was not well developed. Its usual fibrogranular appearance of uniform thickness was replaced by patchy intermittent amorphous material, and the cells of the secondary mesenchyme contained numerous large rounded secretion granules. In their study in which LACA was administered for a 5-day period to chick embryos, Lane

et al. (1971) found that LACA consistently affected the accumulation of collagen more so than other proteins. The collagen produced had a decreased number of cross-striated fibrils; otherwise the physical and chemical properties appeared to be similar to normal collagen. Therefore, they postulated that a proline analog might be developed which would be useful in controlling collagen synthesis in clinical conditions such as scleroderma (chronic hardening and shrinking of the connective tissue) and fibrous adhesions.

In another study by Koch (1974) using embryonic mice, explanted rudiments grown in the presence of LACA failed to show the normal developmental alterations. Additionally, extracellular matrix materials were not observed in living cultures; neither dentin nor enamel was seen in histological sections. Exposure to LACA also suppressed new matrix formation in rudiments which had initiated differentiation prior to exposure to the inhibitor. A clear zone was observed separating ameloblasts and odontoblasts. Rats and mice were injected with LACA (500 mg/kg) and were fixed and sections examined under the microscope (Sudo et al., 1978). The odontoblastic layer showed little irregular arrangement and reduction in length. With the EM, the young odontoblasts showed vesiculation of r-ER as well as the presence of intracisternal materials within the r-ER. An increase in cytosegresomes and dense bodies was also observed. However, Sudo et al. (1977) observed that LACA caused skeletal

abnormalities in the lumbar and sacral vertebrae in mice, and no inhibitory effect on odontogenesis was noted. By injecting rats with 100-300 mg/kg from the 0-day and 100 mg/kg from 8-day to 10-day, Nagai et al. (1976) showed that in rats treated with 100 mg/kg the fetuses were externally malformed and had a reduction in ossification of the sternebrae. Treatment with 300 mg/kg gave a high fetal mortality and growth retardation in the surviving fetuses. Ossification was retarded in the supraocciput, sternebrae, and coccygeal vertebrae. A radiopaque layer was seen to appear in the dentine of treated rats and mice (Kambara et al., 1976). This layer was considered to have been formed when LACA was administered. In contrast, a radiolucent layer was detected in the enamel. They suggested this might have been formed at the time of drug administration. They also pointed out that rats were more susceptible to this drug than mice; that in rats dentine formation was retarded following injection, whereas enamel formation recovered well. The effects of the drug on mice were transitory, and both dentine and enamel were recovered soon after the drug administion.

In their studies with Swiss mice, Ruch et al. (1974) showed that in buds removed and cultured in the presence of LACA, the apical odontoblasts did not differentiate morphologically and did not secrete predentin. They showed that the effects of LACA were reversible, and this was demonstrated

by cultivating the buds for four days in LACA followed by two days in control medium. At the end of treatment, the apical odontoblasts appeared to be morphologically and functionally differentiated. They also showed that in buds cultured for two days in the presence of LACA, there were less morphologically-differentiated odontoblasts, and also no picrofuchsin-positive substance (collagen) was found. Odontoblasts were rounded after four days culture and no picrofuchsin-positive substance was found. The apical ameloblasts (i.e., those which have become post-mitotic) were not polarized and, consequently, LACA prevented excretion of collagen by odontoblasts. A study by Strudel (1975) showed that LACA inhibited the secretion of the periaxial extracellular material, resulting in a lack of several vertebrae. In young vertebral primordial cultured on LACA, the myotome cells differentiated into myoblasts, whereas the sclerotome cells remained undifferentiated. In older primordia, the sclerotome cells gave rise to abnormal cartilage but the myotome cells seldom differentiated. Treatment at 9 days of incubation showed that embryonic growth was retarded by LACA (Brian, 1978). It was also shown from this study that secondary chondrogenesis of the quadratojugal, a membrane bone of the skull, was inhibited by treatment of the whole embryo with LACA.

The large increase in collagen observed in the livers of patients with hepatic cirrhosis (Popper and Udenfriend,

1970), or in the livers of rats made cirrhotic by chronic administration of CCl_4 (Huberman et al., 1969; Rojkind and Diaz de Leon, 1970), or of ethanol (Feinman and Lieber, 1972) is largely due to de novo synthesis. In these cases, increase in collagen is accompanied by a parallel increase in the pool of free proline and a decrease in the pool of free glutamic acid. The increase in proline occurs principally in the liver (Kershenobich et al., 1970) and is due in part to more active synthesis from glutamic acid. Rojkind (1973) showed that rats treated with LACA developed a lesser degree of cirrhosis and their livers contained a smaller pool of free proline when compared with the untreated cirrhotic rats. It was suggested that the decrease in the pool of free proline was due to the fact that either LACA inhibited biosynthesis or interfered with the active transport of this amino acid into hepatic fibroblasts. It was also demonstrated that when cirrhogenic doses of CCl_4 were administered to rats concurrently, LACA caused a decreased incorporation of labeled proline into collagen.

Another study by Kershenobich and Rojkind (1973) showed that the livers of CCl_4-treated animals contained a large increase in collagen and reticular fibrils, cell necrosis fatty infiltrations, and distortion of liver architecture, but the livers of animals treated with LACA in addition to CCl_4 showed a lesser degree of fibrosis but contained the same degree of fatty infiltration and liver cells necrosis.

They further stated that the liver function in the rats receiving only CCl_4 was impaired, as was observed by the increased retention of conjugated bilirubin and the low levels of serum albumin. But they pointed out that serum conjugated bilirubin levels in rats treated with 100 or 200 μ moles of LACA were significantly lower, as compared with the values obtained in rats treated only with CCl_4. Finally, this study showed that in animals treated with 100 μ moles of LACA, the total albumin decreased, whereas in animals treated with 200 μ moles of LACA the albumin values were close to normal.

When freshly isolated cells from chick embryo tendon were incubated in the presence of azetidine-2-carboxylic acid, Dehm and Prockop (1971) demonstrated the analog decreased the relative amount of [^{14}C] hydroxyproline synthesized in the presence of the analog. Livers of mice with hepatosplenic schistosomiasis (Bilharziasis) contain 20 times more collagen than normal, and this hepatic fibrosis is due to an inflammatory granulomatous host response to Schistosoma mansoni ova in portal tracts, rather than from direct liver cell injury, as with carbon tetrachloride induced liver fibrosis (Dunn et al., 1977). These investigators demonstrated that the formation of labeled protein-bound hydroxyproline decreased sharply as the concentration of the proline analog (LACA) in the medium was increased. They further showed that at 100 mM LACA, production of labeled hydroxyproline was inhibited by 95%. A parallel effect of the

analog was noted on incorporation of labeled proline into total liver protein.

Histological examination of the livers of animals treated with CCl_4 showed a large increase in collagen and reticular fibers, cell necrosis, fatty infiltration, and distortion of liver architecture (Rojkind and Kershenobich, 1975). It was further demonstrated that the livers of animals treated with colchicine plus CCl_4 showed a lesser degree of fibrosis, but the same degree of necrosis and fatty infiltration. They further showed that the livers of rats treated with LACA after CCl_4 administration did not show any improvement in fibrosis or fatty infiltration. In this study, they concluded that the specific activity of collagen hydroxy-proline in rats treated with colchicine after CCl_4 administration was very low and similar results were obtained with animals treated with LACA after CCl_4 administration.

Using mouse embryonic lung, Alescio (1973) showed that LACA inhibited lung morphogenesis. It was shown that at low concentrations of the analog, a sharp decline of the number of newly formed germinal buds was induced. It was further observed that DNA and protein were unequally reduced by the time the treatment was terminated. The protein concentration was seen to be more depressed, with subsequent rise of the DNA/protein ratio. The results showed that epithelial budding was very sensitive to treatment with the analog.

The effect of LACA on the free-living nematode Panagrellus silusiae was demonstrated by Leushner and Pasternak (1978). It was pointed out that morphometrically, the worms showed no evidence of an adverse response to the long-term presence of LACA in the growth media. Incorporation of labeled proline into protein was blocked by the analog, whereas the uptake of radiolabeled leucine into polypeptides was unaffected. Conversely, the uptake of labeled azetidine carboxylate was antagonized only by proline. They further pointed out that the incorporation of labeled analog into collagen during post-embryonic development paralleled the discontinuous pattern of collagen biosynthesis in untreated worms. Cuticular collagens became labeled after incubation with [^{14}C] azetidine carboxylate. From their results, it was suggested that the analog incorporated into collagenous and non-collagenous proteins.

To examine the effects of LACA on glycosylations of collagen in chick embryo tendon cells, Oikarinen et al. (1976) incubated the isolated cells in the presence of LACA and observed that the degree of glycosylations of hydroxylysine during the first 10 min of the chase period was identical with that in cells incubated without the compound. They stated that glycosylations continued in the presence of LACA for 60 min, whereas no additional glycosylations took place in the control cells after 10 min. As a result, the collagen

synthesized in the presence of this compound contained more carbohydrate than did the collagen synthesized by the control cells. They also indicated that LACA did not increase the collagen glycosyltransferase activities in the tendon cells or the rate of glycosylation reactions when added directly to the enzyme incubation mixture. They also observed that control experiments with colchicine showed that the delay in collagen secretion in the presence of LACA did in itself affect the degree of glycosylations of collagen. It was, therefore, suggested that the increased glycosylations were due to inhibition of the collagen triple-helix formation which occurs in the presence of LACA.

The LACA was also shown to prevent the formation of fibrils in cultured epithelial primordia and in cultures of whole thyroids (Hilfer and Pakstis, 1977). Furthermore, the mesenchymal cells did not invade when whole thyroid primordia were cultured in the presence of the drug. However, it was demonstrated that the effects were reversed by incubation with equimolar or greater amounts of L-proline added to the medium along with the drug.

Studies by Trasko et al. (1976) showed that the proline analog was incorporated into hemoglobin S (sickle hemoglobin) in vitro. Sickle erythrocytes from patients with sickle cell anemia, incubated with L-[^{3}H] azetidine-2-carboxylate, synthesized radiolabeled hemoglobin which when isolated from cell lysates co-chromatographed with hemoglobin S on DEAE

cellulose columns. It was also shown that the α/β ratio of azetidine carboxylate incorporation into the globin chains of sickle hemoglobin was 0.94, which was consistent with the presence of four proline residues in each polypeptide chain. It was shown further that azetidine carboxylate did not inhibit uptake of radiolabeled proline by sickle erythrocytes, suggesting that the homolog does not adversely affect amino acid transport in the cells. The incorporation into hemoglobin in rabbit reticulocytes in vitro was demonstrated by Baum et al. (1973). This investigation was the first to show that LACA was incorporated into mammalian protein.

CHAPTER III

MATERIALS AND METHODS

Fertile eggs of White Leghorn chickens were obtained from H. and E. Rabbitery and were incubated in a humid atmosphere 37.8 C. Prior to incubation the eggs were swabbed with 70% ethyl alcohol. The fertilized eggs were divided into four groups: A, B, C, and D. Group A was comprised of controls and Groups B, C, and D comprised of experimentals. All of the groups were injected either prior to incubation or at 24 hr incubation. Group A, which comprised the controls, was given three series of injections: pre-incubation, 48 hr, and 96 hr with 0.6 cc of 0.85% normal saline; Group B was injected once with 0.6 cc L-azetidine-2-carboxylic acid (LACA; obtained from Sigma, St. Louis, Missouri); Group C was injected two times at pre-incubation and at 48 hr with 0.6 cc LACA; Group D was injected three times at pre-incubation, 48 hr, and 96 hr with 0.6 cc LACA. The eggs were sacrificed at 7-day, 9-day, 11-day incubation, and were fixed, respectively. The embryos were fixed in 10% buffered formalin for 24 hr (Hollister, 1934; Hood and Neill, 1948; Youngpeter, 1964), followed by 2% potassium hydroxide (KOH) for 24 hr, and were then transferred to an alizarine working solution and back into KOH for 24 hr each. They were then placed in three cleaning solutions--I, II, and III--for 24 hr each:

Solution I: glycerine 20 parts, 4% potassium hydroxide 3 parts, distilled water 77 parts; Solution II: glycerine 50 parts, 4% potassium hydroxide (KOH) 3 parts, distilled water 47 parts; Solution III: glycerine 75 parts, distilled water 25 parts. This method was employed for the demonstration of calcium deposition in chick bones.

In preparation for paraffin embedding, the embryos were fixed in Bouin's fluid for 24 hr. They were dehydrated in a series of increasing strength alcohol solutions (50%-100%) 30 min each; alcohol-xylene (ratio 1:1) for 30 min (two changes of 15 min each); xylene-paraffin solutions (ratio 1:1), and placed in an oven at 56 C 1 hr, and pure paraffin solution at 56 C for 1½ to 2 hr, after which the embryos were embedded in pure paraffin. Sections were made at 10-μ on the 820 Spencer microtome and mounted on glass slides. The slides were deparaffinized in xylene for 1 min and dried. In order to remove artefact pigment (the formalin derived pigment seen on the tissues), the slides were again, after drying, placed in xylene for 2 min.

Neutral-acid Mucopolysaccharides Analysis

Two techniques were employed for the analysis of two types of glycosaminoglycans (acid mucopolysaccharides). One was Alcian blue-PAS method (Mowry, 1956). Solutions required were:

1. Alcian blue (pH 2.5): Alcian blue 1 gm, 3% acetic

acid and distilled H_2O 100 ml.

2. Alcian blue (pH 0.5): Alcian blue 1 gm, 0.5 N hydrochloric acid and distilled H_2) 100 ml.

Solutions were filtered before use and stable for approximately 2 weeks. The sections were hydrated through a series of decreasing strength solutions: xylene 3 min (two changes), xylene-alcohol (ratio 1:1) 3 min, 100% alcohol 3 min, 95% alcohol 3 min, 70% alcohol 2 min. They were stained in Alcian blue solution for 10 min; washed in distilled water; oxidized in 1% periodic acid for 5 min. They were again washed well in distilled water; placed in Periodic Acid Schiff's (PAS) reagent for 10 min; and washed in running tap water for 10 min. They were counterstained lightly in Mayer's haematoxylin and were differentiated in 1% acid alcohol. The slides were dehydrated through graded alcohols to xylene.

Hyaluronic-chondroitin Analysis

This technique was employed to analyse hyaluronic and chondroitin sulfates, and it has been found by other investigators to give excellent analysis of hyaluronic and chondroitin sulfates (Spicer, 1965; Leppi and Spicer, 1967). Diamine solution: N, N-dimethyl-M-phenylenediamine dihydrochloride 120 mg, N, N-dimethyl-p-phenylenediamine dihydrochloride 20 mg. These salts were dissolved in 50 ml of distilled water and then added 40% ferric chloride (1.4 ml);

the pH of the solution was 1.5. The Alcian blue solution (pH 2.5): Alcian blue 1 gm; 3% acetic acid; distilled water 100 ml. The sections were rehydrated through a series of decreasing strength alcohols to xylene, stained in diamine solution for 18 hr and washed in water. They were stained in Alcian blue solution for 10 min and again washed in water. They were then dehydrated through graded alcohols to xylene.

Photographs of whole embryos were made with the Kodak Ektagraph Visualmaker, and the photomicrographs of sections with an American Optical Expostar Assembly.

CHAPTER IV

EXPERIMENTAL OBSERVATIONS

Macroscopic Observations

The control embryos were administered 0.6 cc normal saline three times--at pre-incubation, 48 hr, and 96 hr. The experimental Group I received 0.6 cc L-azetidine-2-carboxylic acid (LACA) one time (pre-incubation), Group II two times (pre-incubation and 48 hr), and Group III three times (pre-incubation, 48 hr, and 96 hr). The embryos for macroscopic studies were sacrificed on days 7, 9, and 11. They were treated in Alizarin Red S for calcium deposition.

Examining the 7 day control and experimental Group I embroys showed that calcium formed an orange-red dye lake with the dye Alizarin Red S. The formation of the orange-red dye lake (calcification) which is spotty was only observed in the metatarsus, tibiofibula, femur, and the anterior regions of the radioulna. At this age there was not any orange-red lake observed in the phalanges, the metacarpalia, and cranio-vertebral regions in both the controls and the experimental Group I (Figs. 1, 2).

The experimental Group II revealed very little calcification in the metatarsus, tibiofibula, the femur, or the radioulna regions. Examination of the phalanges, the metacarpalia, and the cranio-vertebral regions showed no

calcification (Fig. 3). Group III showed little or no calcification in any of the regions (Fig. 4).

The 9-day-old embryos of both the controls and the experimental Group I showed that the formation of the orange-red dye lake was faint in the phalanges, vertebral, and the cranial regions. But looking at the ribs, the metatarsus, tibiofibula, and the radioulna regions, the formation of calcium deposits was observed to be greater in 9-day embryos than in the 7-day-old embryos (Figs. 5, 6). Experimental Groups II and III of 9-day-old embryos revealed that calcification was comparable to that of the 7-day-old control and experimental Group I embryos. These deposits appeared patchy and were observed in the metatarsus, ribs, tibiofibula, and radioulna regions. They were faint in the cranio-vertebral regions, and were almost absent in the phalanges and metacarpalia regions of these two groups (Figs. 7, 8).

Examination of the 11-day-old embryos revealed that calcification was well advanced in the metatarsus, tibiofibula, femur, ribs, and the cranio-vertebral regions of controls, and experimental Groups I and II; however, in the phalanges and metacarpalia regions calcification was still patchy in these groups (Figs. 9, 10, 11). Experimental Group III revealed that calcification was comparable to that of the controls and experimental Group I of the 9-day-old embryos. Calcification was observed in the metatarsus,

tibiofibula, femur, and ribs; however, in the phalanges, metacarpalia, and cranio-vertebral regions, it was not observed (Fig. 12).

Light Microscopic Observations

A histological examination of embryos was conducted to ascertain the effects of LACA on calcium deposition. Control and experimental embryos in Group I (receiving 0.6 cc LACA) showed a heavy orange-red dye (indicating calcium deposits) in the perichondral regions, whereas very little or none was observed within the cartilage (Figs. 13, 14). But in Group II embryos which received 0.6 cc LACA two times (pre-incubation and 48 hr) and Group III which received three injections of 0.6 cc LACA (pre-incubation, 48 hr, and 96 hr), the deposition of calcium in the perichondral regions was ranged from zero to mild; hardly any calcium deposition was observed within the cartilage (Figs. 15, 16). This condition was observed principally in embryos sacrificed at 7 days. Although this condition was applicable to 9-day embryos, a slight difference was noted particularly among the control and experimental Group I. Among these groups a heavier localization or deposition of calcium was observed in the perichondral regions, and a slight one in the cartilage (Figs. 17, 18). Group II, which received 2 injections of LACA, exhibited a mild to slight deposition of calcium in the perichondral regions and the cartilage

Fig. 1. Photograph of 7-day-old embryo. A control embryo. Orange-red dye lake shows calcium deposit in the limbs, but none observed in the cranio-vertebral regions or the phalanges 10x.

Fig. 2. Photograph of 7-day-old embryo 0.6 cc LACA-treated (1x) embryo. Orange-red dye lake shows calcium deposition in the limbs. No calcium deposition was observed in the cranio-vertebral regions or the phalanges 10x.


1


2

Fig. 3. Photograph of 7-day-old embryo 0.6 cc LACA-treated (2x) embryo. Very little calcium deposition observed in the limbs and none observed in the cranio-vertebral regions, or phalanges, and limb buds are short 10x.

Fig. 4. Photograph of 7-day-old embryo 0.6 cc LACA-treated (3x) embryo. Very little calcium deposition was observed in the limb buds. None was observed in the cranio-vertebral regions or phalanges 10x.


3


4

Fig. 5. Photograph of 9-day-old embryo. A control embryo, (showing calcium deposition in limbs, and vertebral region. Very little was observed in the phalanges and cranial regions 10x).

Fig. 6. Photograph of 9-day-old embryo. 0.6 cc LACA-treated (1x) embryo (showing calcium deposition in the limbs, and the vertebral regions. Very little was observed in phalanges, the neck, or cranial regions 10x).

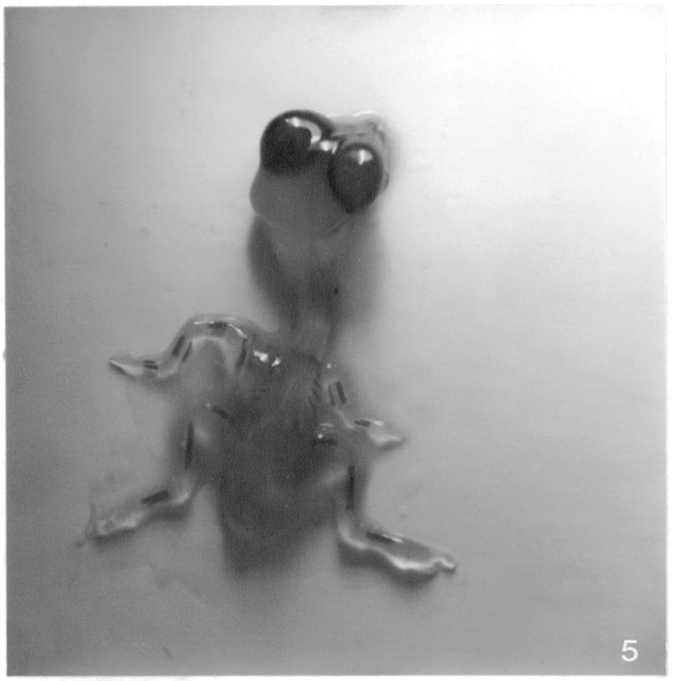
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Fig. 7. Photograph of 9-day-old embryo. 0.6 cc LACA-treated (2x) embryo (showing little calcium deposition in the limb buds, and vertebral regions. None observed in phalanges or cranial regions 10x).

Fig. 8. Photograph of 9-day-old embryo 0.6 cc LACA-treated (3x) embryo (showing little calcium deposition in the limbs. Very little or none observed in the vertebral region. None was observed in phalanges or cranial regions 10x).


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Fig. 9. Photograph of 11-day-old embryo. A control embryo, (showing calcium deposition in limbs, and cranio-vertebral regions 10x).

Fig. 10. Photograph of 11-day-old embryo 0.6 cc LACA-treated (1x) embryo (showing calcium deposition in limbs and cranio-vertebral regions 10x).


9


10

Fig. 11. Photograph of 11-day-old embryo. 0.6 cc LACA-treated (2x) embryo showing deposition of calcium in limbs and cranio-vertebral regions 10x.

Fig. 12. Photograph of 11-day-old embryo. 0.6 cc LACA-treated (3x) embryo (showing calcium deposition in limbs and vertebral regions. None was observed in phalanges, neck, or cranial regions 10x).


11


12

exhibited only a slight deposition (Fig. 19). Looking at the perichondral regions of those embryos receiving 3 injections (Group III), some regions were observed to be devoid of calcium deposit, whereas some regions were observed to be mildly calcified, while others were slightly calcified (Fig. 20).

The data in Table 1 will show the Alizarin Red S intensity in the control and experimental Groups I, II, and III. Control and experimental Group I showed a mild reaction while Groups II and III varied from slight to mild and no reaction to slight reaction in Group III of day 7 embryos. In day 9 embryos, the reaction was intense in the control and varied from mild to intense in the experimental Group I. Experimental Group II showed a mild reaction whereas experimental Group III showed a slight reaction.

Histochemical Observations

Glycosaminoglycans or acid mycopolysaccharides are divided into two groups: neutral mucopolysaccharides and acid mucopolysaccharides. Acid mucopolysaccharides are further divided into two groups: nonsulfated acid mucopolysaccharides and sulfated acid mucopolysaccharides.

The nonsulfated group includes hyaluronic acid and chondroitin. The sulfated includes chondroitin 4-sulfate (chondroitin sulfate A), chondroitin-6 sulfate (chondroitin

Table 1. The effects of L-azetidine-2-carboxylic acid (1 mg/ml) on calcium deposition during skeletogenesis.

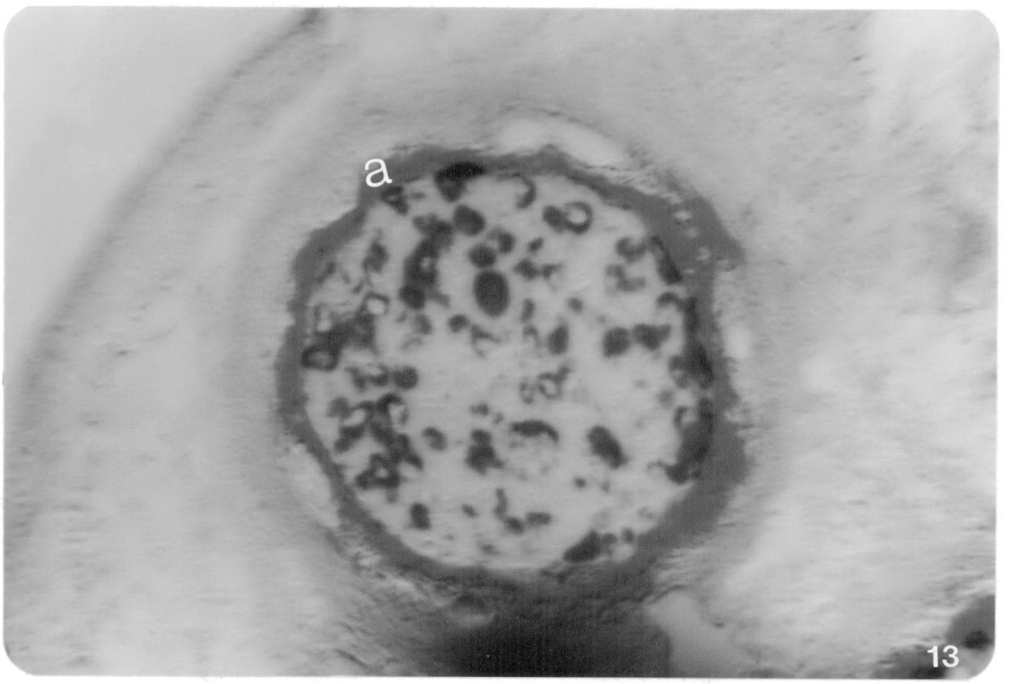
Group	# Embryos Injected	Dosage	# Injections Given	Alizarin Red S Intensity Age (Days) 7	9
Controls (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3*	++	+++
Experimentals					
Group I (Injected Pre-incubation)	245	0.6 cc	1	++	++/+++
Group II (Injected Pre-incubation and 48 hr)	245	0.6 cc	2	+/++	++
Group III (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3	0/+	+

+++ Intense Reaction; ++ Mild Reaction; + Slight Reaction; 0 No Reaction.

*Controls were injected Pre-incubation, Pre-incubation plus 48 hr, Pre-incubation, 48 hr, and 96 hr. Since no differences were noted, the triply injected are cited here.

Fig. 13. Cross-section of metatarsus of 7-day control embryo (showing orange-red dye lake (calcium deposition) formation at perichondral regions of cartilage (a). Little or none was observed within cartilage 10x).

Fig. 14. Cross-section of metatarsus of 7-day 0.6 cc LACA-treated (1x) embryo (showing calcification (orange-red dye lake formation), at perichondral regions of cartilage (a). Little or none was observed within cartilage and none within connective tissue 10x).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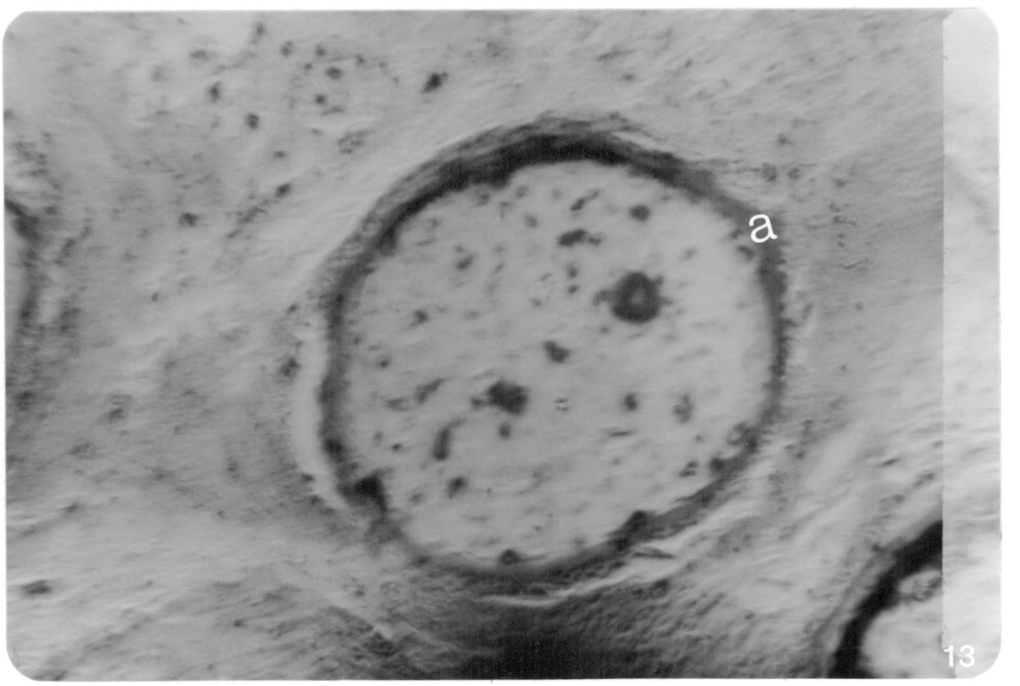
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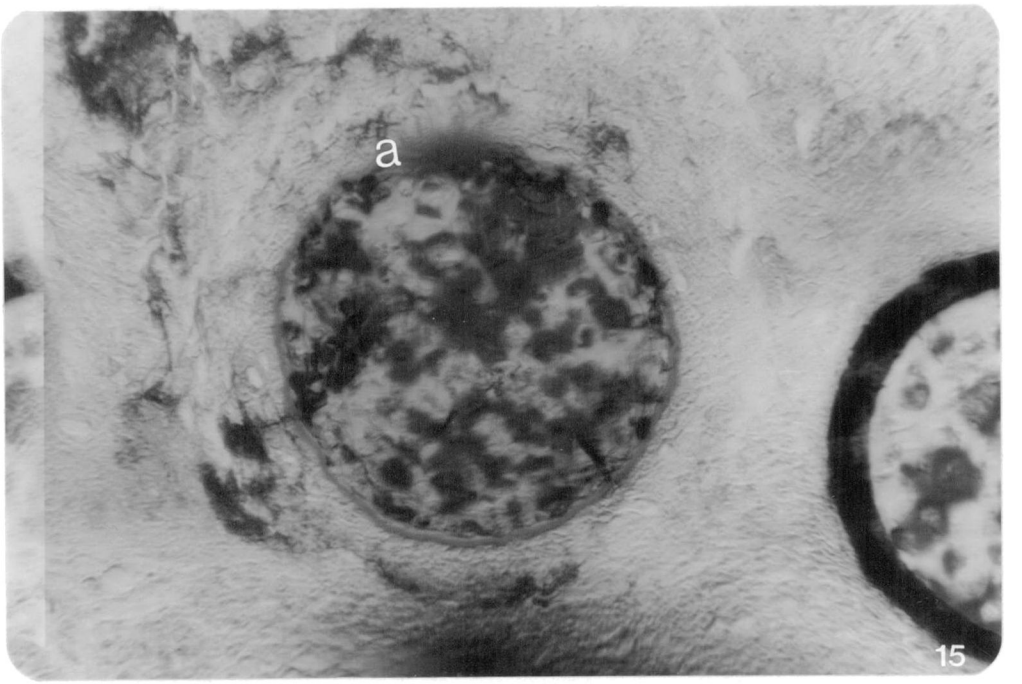
Fig. 15. Cross-section of metatarsus of 7-day 0.6 cc LACA-treated (2x) embryo (showing little calcification at perichondral regions of cartilage (a). None was observed within cartilage 10x).

Fig. 16. Cross-section of metatarsus of 7-day 0.6 cc LACA-treated (3x) embryo (showing very little calcification at perichondral regions of cartilage (a). None was observed within cartilage 10x).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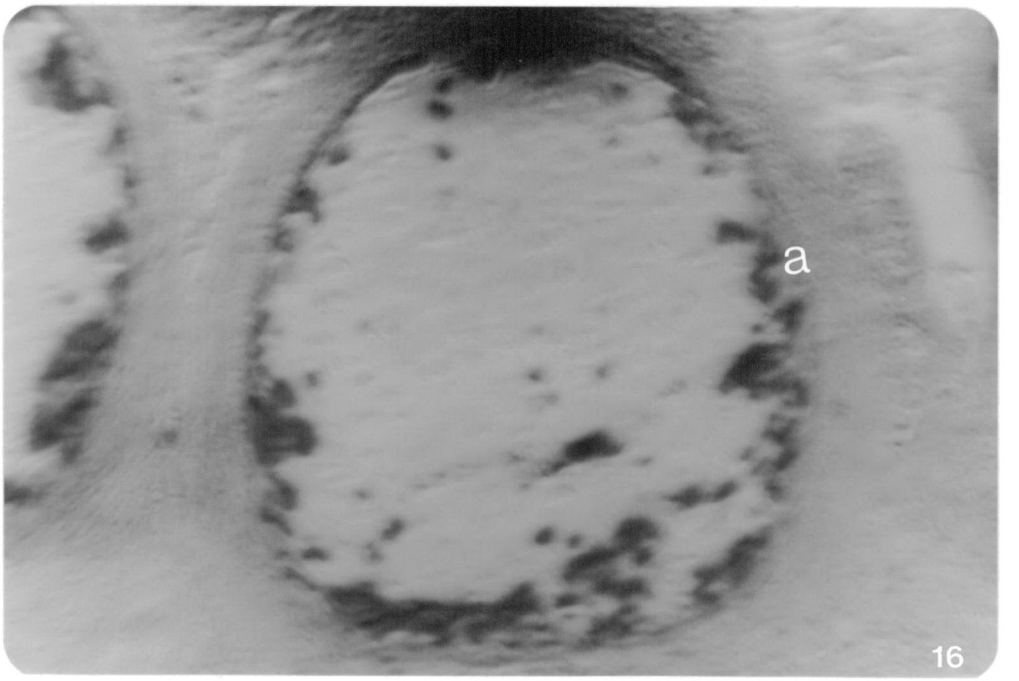
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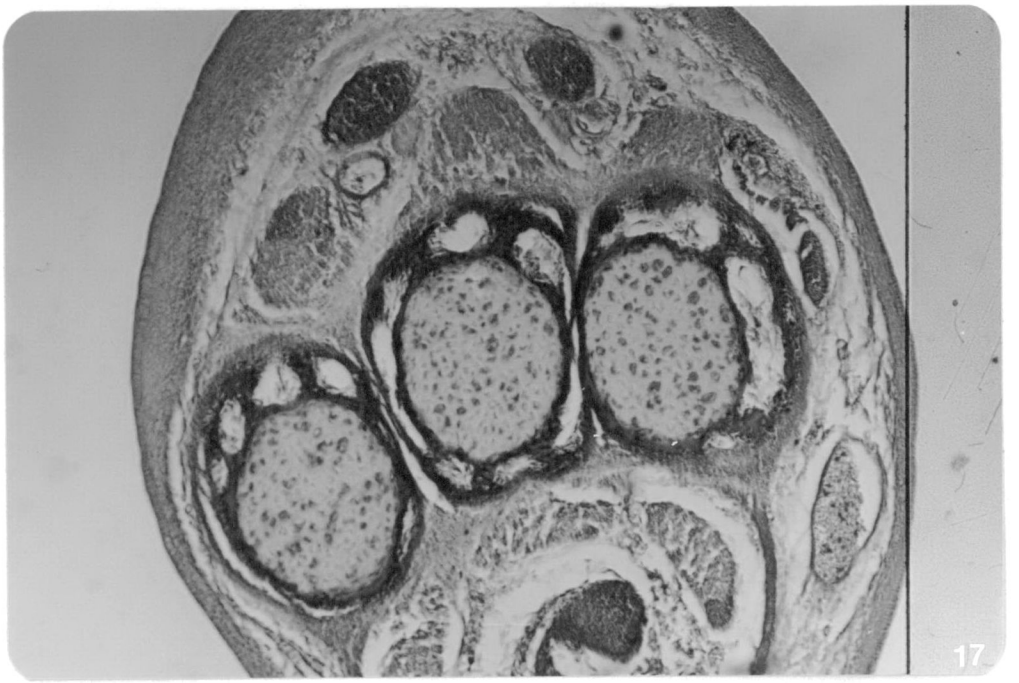
Fig. 17. Cross-section of metatarsus of 9-day control embryo (showing calcification at the perichondral regions. Little or none was observed within cartilage 10x).

Fig. 18. Cross-section of metatarsus of 9-day 0.6 cc LACA-treated (1x) embryo (showing calcification at perichondral regions. Little or none was observed within cartilage 10x).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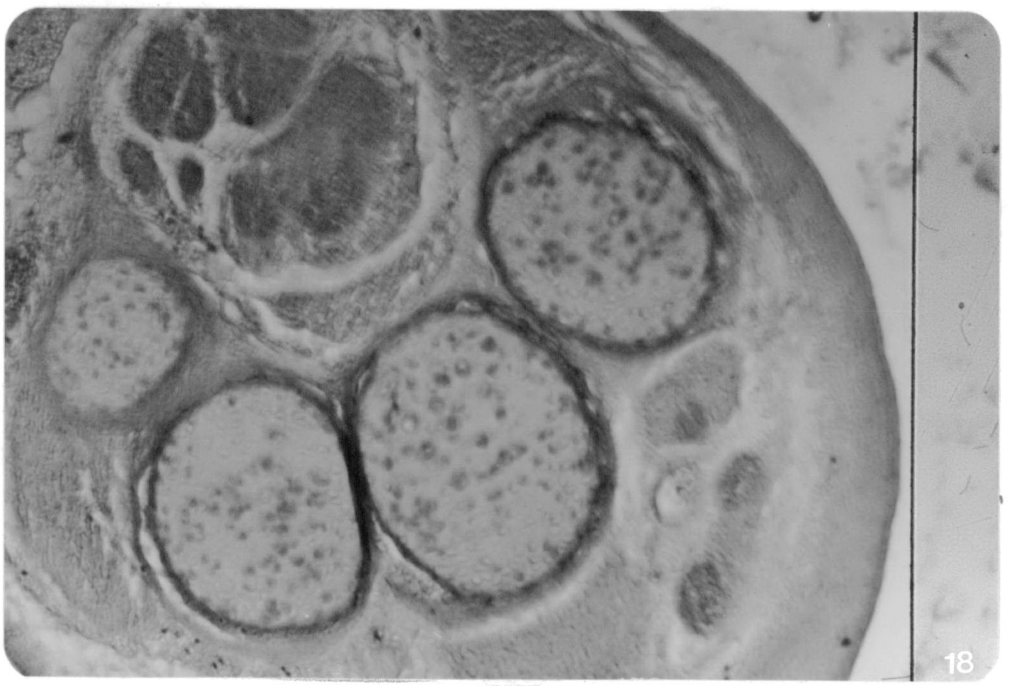
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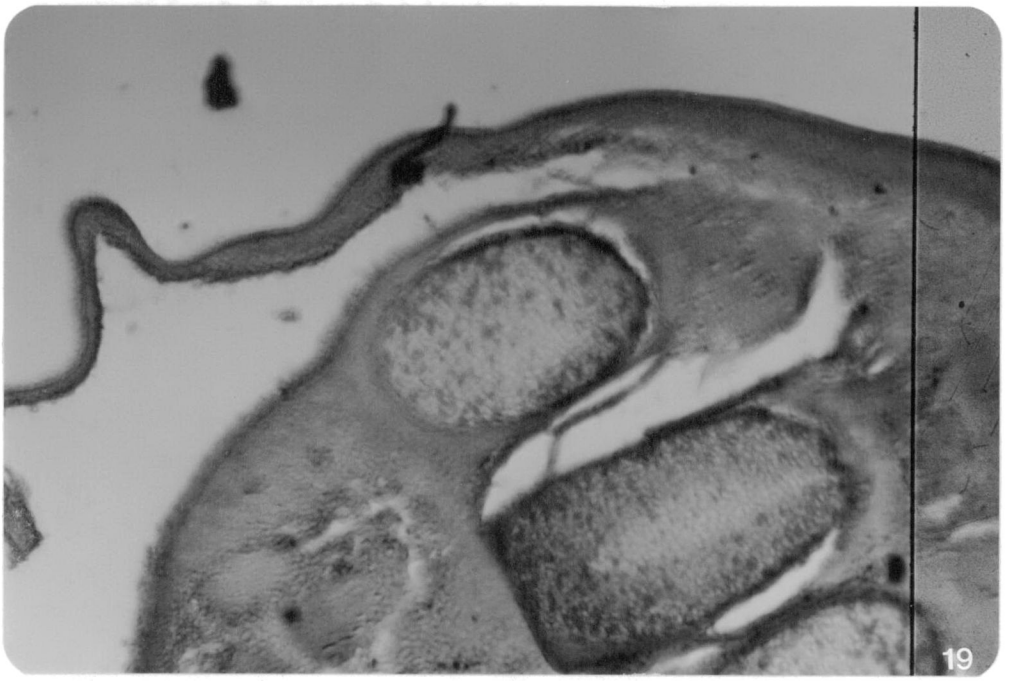
Fig. 19. Cross-section of metatarsus of 9-day 0.6 cc LACA-treated (2x) embryo (showing little calcification at perichondral regions 10x).

Fig. 20. Cross-section of metatarsus of 9-day 0.6 cc LACA-treated (3x) embryo (showing very little calcification at perichondral regions 10x).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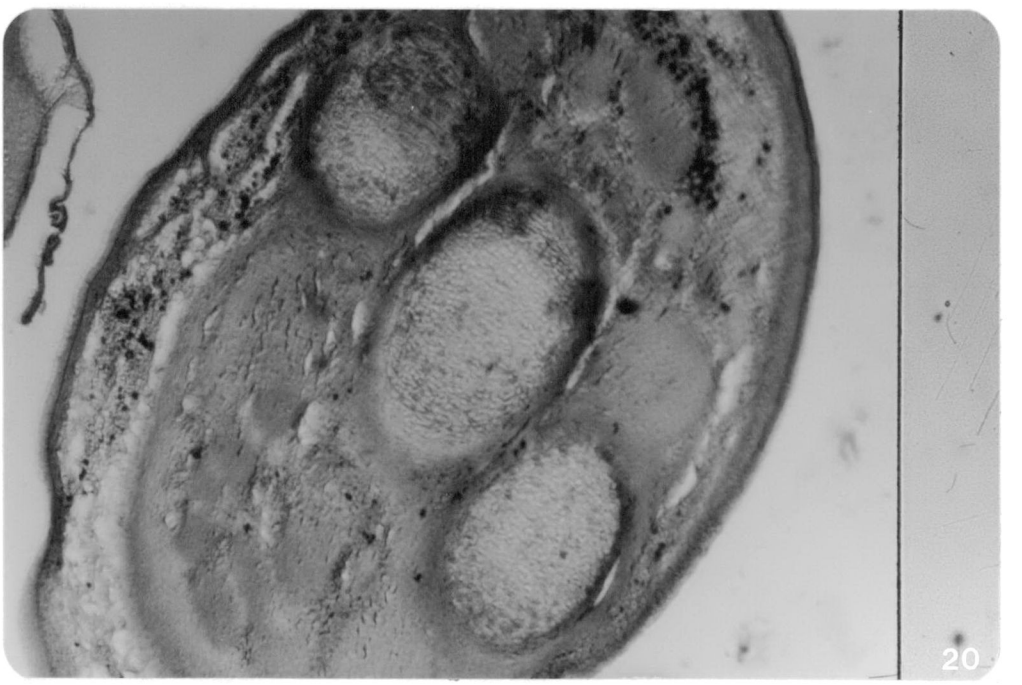
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sulfate C), dermatan sulfate (chondroitin sulfate B), and Keratan sulfate (Kerato sulfate).

Periodic Acid Schiff's (PAS)-Alcian Blue Reactions

In PAS-Alcian blue preparations, the neutral acid mucopolysaccharides are PAS positive orange-red (acidophilic), whereas nonsulfated and sulfated acid mucopolysaccharides are Alcian blue positive (basophilic) and PAS negative (Bancroft, 1975). Table 2 shows that control embryos that were administered 0.6 cc normal saline three times--at pre-incubation, 48 hr, and 96 hr--and experimental Group I which received 0.6 cc of LACA one time--at pre-incubation--exhibited the same PAS-Alcian blue intensity. Experimental Groups II and III exhibited the same intensity at age 7-day or 9-day.

Table 2 also shows that at day 7 in both the control and the experimental Group I, there was a mild reaction for neutral mucopolysaccharides, whereas in experimental Groups II and III the reaction of PAS-Alcian blue (pH 2.5) with neutral acid mucopolysaccharides was intense. At the same age, the reaction of PAS-Alcian blue (pH 2.5) with connective tissue nonsulfated acid mucopolysaccharides, and connective tissue sulfated acid mucopolysaccharides, PAS-Alcian blue (pH 1.0 or pH 0.5) showed a mild to intense reaction in both control and experimental Group I. But the reactions in experimental Groups II and III ranged from slight to mild, and

Table 2. The effects of L-azetidine-2-carboxylic acid (1 mg/ml) on neutral, sulfated, and non-sulfated mucopolysaccharides during skeletogenesis.

Group	# Embryos Injected	Dosage Given	# Injections Given	Alcian blue-PAS Indications: Neutral Mucopol. (Days) 7	9	Conn. Tiss. Sulf. Acid Mucopol. (Days) 7	9	Non Conn. Tiss. Sulf. Acid Mucopol. (Days) 7	9
Controls (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3*	++	++	++/+++	+++	++/+++	+++
Experimentals									
Group I (Injected Pre-incubation)	245	0.6 cc	1	++	++	++/+++	+++	++/+++	+++
Group II (Injected Pre-incubation, 48 hr)	245	0.6 cc	2	+++	+++	+/++	+/++	0/+	+/++
Group III (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3	+++	+++	0/+	+/++	0/+	+/++

+++ Intense Reaction; ++ Mild Reaction; + Slight Reaction; 0 No Reaction.

*Controls were injected pre-incubation, pre-incubation plus 48 hr, pre-incubation, 48 hr, and 96 hr. Since no differences were noted, the triply injected are cited here.

no reaction to slight. At age 9 days the reaction intensity of neutral acid mucopolysaccharides (Alcian blue pH 2.5) for both control and experimental Group I was mild, whereas Groups II and III showed an intense reaction. The reaction of connective tissue nonsulfated acid mucopolysaccharides (Alcian blue pH 2.5) and connective tissue sulfated acid mucopolysaccharides (Alcian blue pH 1.0 or pH 0.5) was intense in both control and experimental Group I. The reaction in experimental Groups II and III was slight to mild.

In order to analyze the neutral and the acid mucopolysaccharides sections of embryos were treated in Alcian blue-PAS method. The 7-day control and Group I embryos showed wide distribution of basophilia (blue-green) which indicates acid mucopolysaccharides. Acid mucopolysaccharides were observed in the matrix of the cartilage, especially in areas surrounding mesenchymal cells; but most of the areas of the matrix within the cartilage showed acidophilia (brown-red), indicative of neutral acid mucopolysaccharides. The perichondral regions also showed neutral acid mucopolysaccharides (Figs. 21, 22). Embryos of Groups II and III showed sparse acid mucopolysaccharides and a heavy accumulation of neutral acid mucopolysaccharides (red stain) (Figs. 23, 24). In some cases the blue-green stain accumulated towards the peripheral regions of the cartilage (Fig. 23). The perichondral regions revealed heavy accumulations of acidophilia. In the femoral region of the 7-day-old embryos, the distribution

of acidophilia and basophilia was different from the phalangial region of the same age. The femoral region showed basophilia, indicating that acid mucopolysaccharides were more prevalent in the control and experimental Group I than in the phalanges of the same Groups II and III. In the femoral region of the control and experimental Group I there was about an equal distribution of basophilia and acidophilia; but the perichondral regions of the cartilage of a femur, like those of phalanges, are acidophilic (Figs. 25, 26). Looking at the femur of embryos receiving 2 injections of LACA (Group II), there was an accumulation of basophilia in some regions of the cartilage while other regions were almost devoid of basophilic stain (Fig. 27).

The embryos that received 3 injections showed a similar condition, but the accumulation of basophilia was much less in all regions of the cartilage. There was more acidophilia in all regions of the cartilage as well as the perichondral regions (Fig. 28).

The metatarsus of a 9-day control embryo showed a predominant distribution of basophilia. Although the distribution of acidophilia was observed in all regions of the cartilage, the accumulation was greater in some regions of the matrix than others. The perichondral regions showed heavier acidophilia, and the connective tissues also showed acidophilia; mature chondrocytes were conspicuous (Fig. 29). The metatarsus of embryos receiving 1 injection showed a

similar distribution of basophilia and acidophilia in the matrix of their cartilages as did those of 9-day controls (Fig. 30). In some regions of the cartilage of this group, the matrix showed acidophilia, while some regions were basophilic. The perichondral regions of this group, as those of control, were heavily acidophilic. The metatarsal regions of embryos receiving 2 injections (Fig. 31) showed accumulations of basophilia mostly in the center of the cartilage around the chondrocytes. The perichonrium and matrix within the cartilage were heavily acidophilic; the connective tissues also showed acidophilia. In the matatarsus of embryos receiving 3 injections, the matrix within the cartilage showed a distribution of acidophilia and basophilia similar to those of Group II, although the distribution of basophilia was observed to be sparse in these groups. The perichondral regions as well as some areas of chondrocytes showed acidophilia (Fig. 32).

The femoral region of 9-day control and Group I embryos had a similar distribution of basophilia. The matrix of the cartilage in these groups contained heavy accumulations of basophilia in some regions while certain other regions showed more acidophilia (Figs. 33, 34). The connective tissues exhibited acidophilia as well. In Group II embryos, the basophilic accumulation was more pronounced in some regions than in some others. Further observation showed that most of the matrix was acidophilic (Fig. 35). Again the

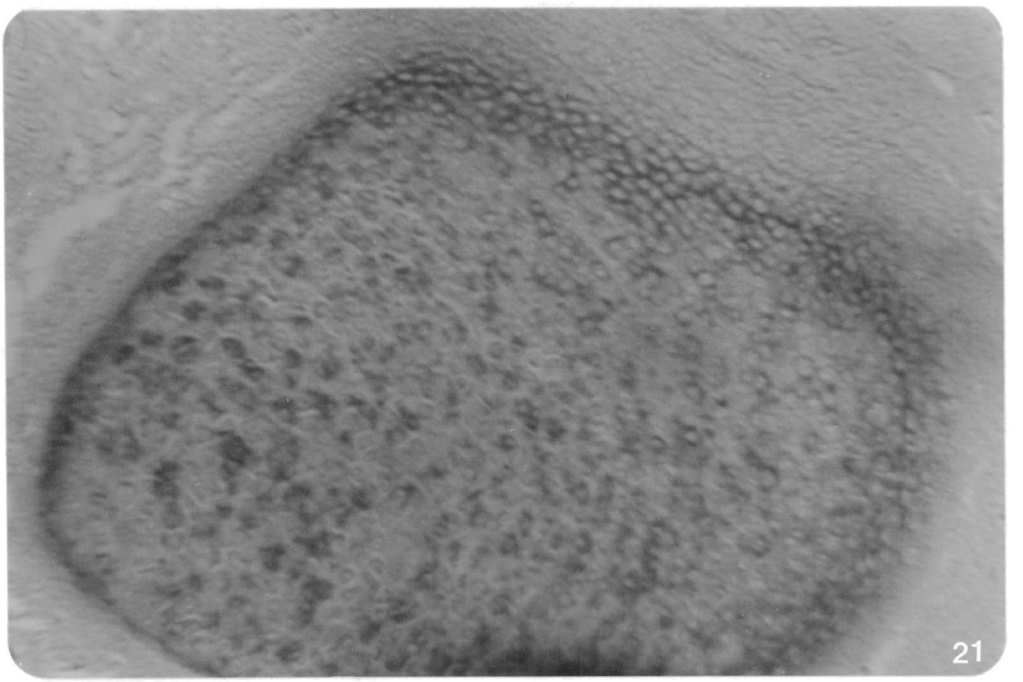
perichondral regions as well as the connective tissues showed only acidophilia. The femoral region of Group III embryos (Fig. 36) indicated that although the basophilia was observed to be even, it was sparse.

Cross-sections through the vertebral regions of 9-day control and experimental Group I embryos are shown in Figs. 37, 38. In these groups the gray matter of the spinal cord was much more acidophilic than the white matter. The lining of the spinal canal showed light acidophilia, and so did the notochord. The neural arch of the vertebra and spinal ganglion showed mostly basophilia (Fig. 37); the acidophilia was sparse. Heavier acidophilia could be detected at the ends of neural arches and the perichondral regions (Fig. 38); however, very sparse acidophilia and heavy basophilia were observed within the cartilage. Again the peripheral regions of the spinal cord showed heavy acidophilia.

In experimental Groups II and III of 9-day embryos, like the control and Group I of the same age, the spinal cord as well as the gray matter of the spinal cord was more acidophilic than the white matter (Figs. 39, 40). The neural arch, however, of Fig. 39 exhibited more basophilia, although sparser than the neural arch of Group III (Fig. 40). Generally in these two groups (II and III), the basophilia was distributed, while acidophilia was heavily distributed over the regions of the neural arch within the cartilage.

Fig. 21. Longitudinal section of phalanx of 7-day control embryo (showing basophilia (blue-green) around mesenchynal cells in matrix. Note certain areas in the matrix are acidophilic (orange-red). Perichondral regions show acidophilia (deep brown-red) (10x).

Fig. 22. Cross-section of phalanx of 7-day 0.6 cc LACA-treated (1x) embryo. (Some areas in the matrix show basophilia while some show acidophilia. Perichondral regions show acidophilia 10x).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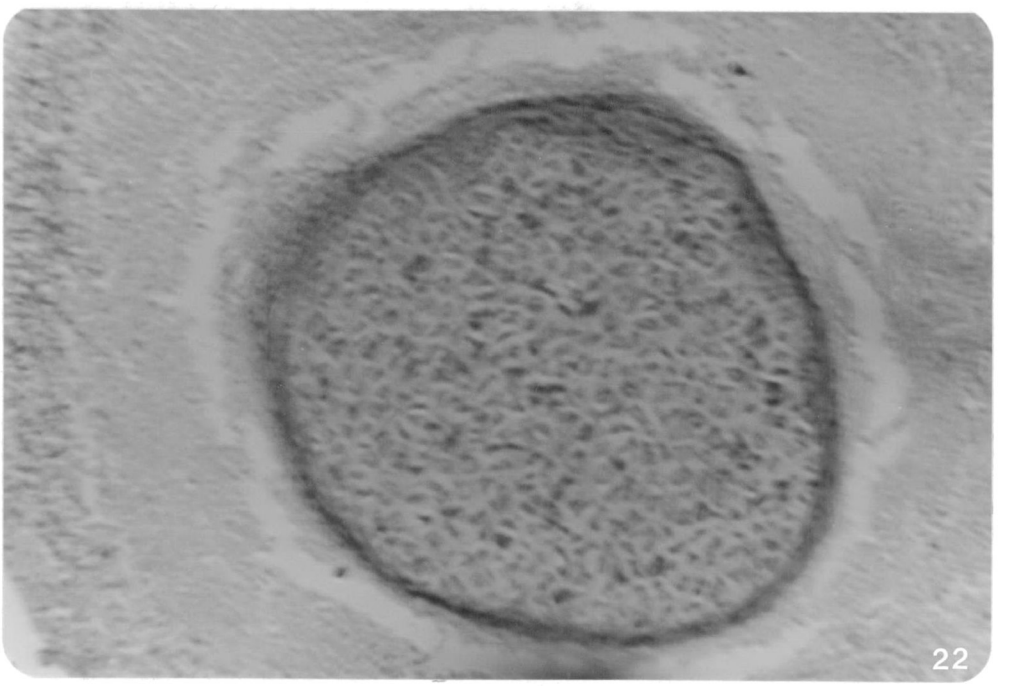
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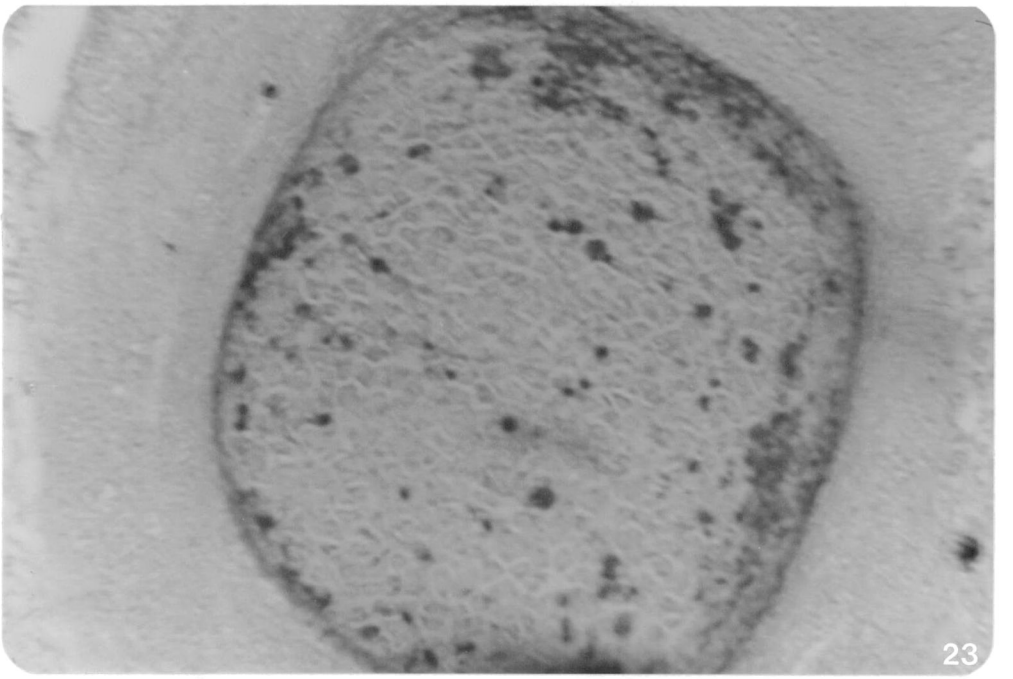
Fig. 23. Cross-section of phalanx of 7-day 0.6 cc LACA-treated (2x) embryo showing basophilia in matrix of peripheral regions of the cartilage. The central areas of the cartilage show mostly acidophilia. The perichondral regions and connective tissue show acidophilia 10x.

Fig. 24. Longitudinal section of phalanx of 7-day 0.6 cc LACA-treated (3x) embryo (showing basophilia in central and few areas of peripheral regions of the cartilage. Most areas of the matrix show acidophilia 10x).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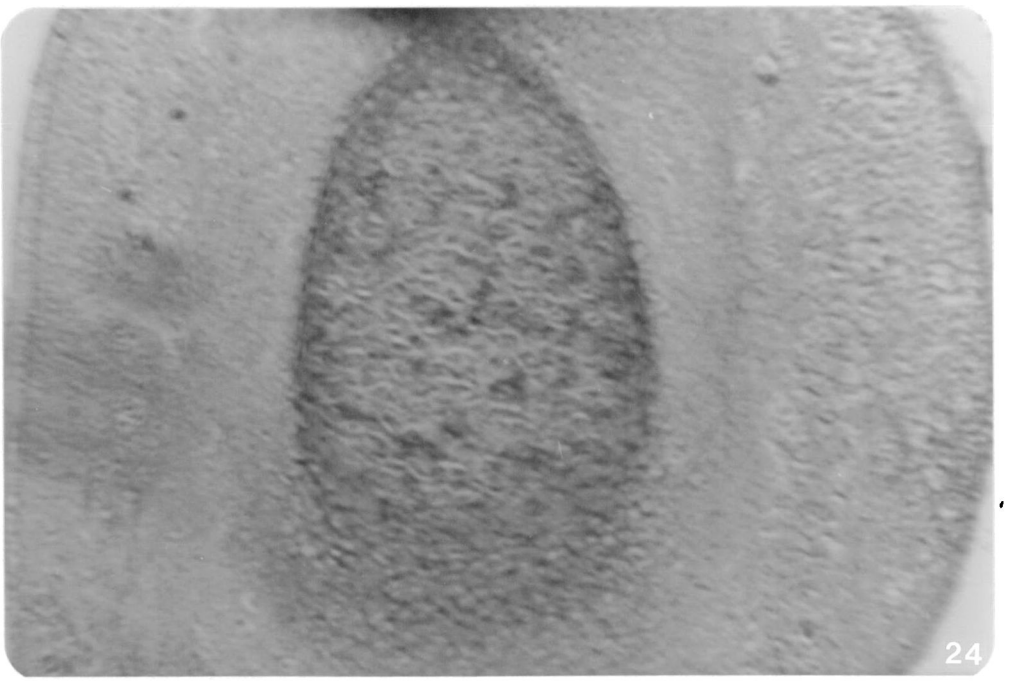
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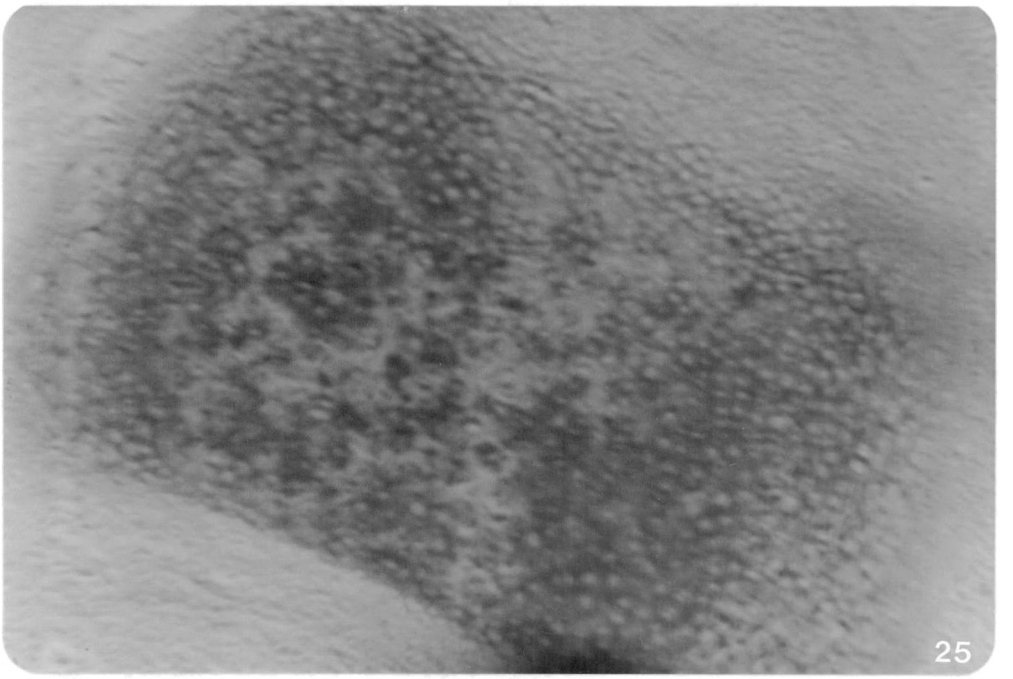
Fig. 25. Longitudinal section of a femoral region of 7-day control embryo (showing basophilia and acidophilia in the matrix. Perichondral regions are acidophilic.

Fig. 26. Longitudinal section of a femoral region of 7-day 0.6 cc LACA-treated (1x) embryo showing areas of matrix that are basophilic and acidophilic.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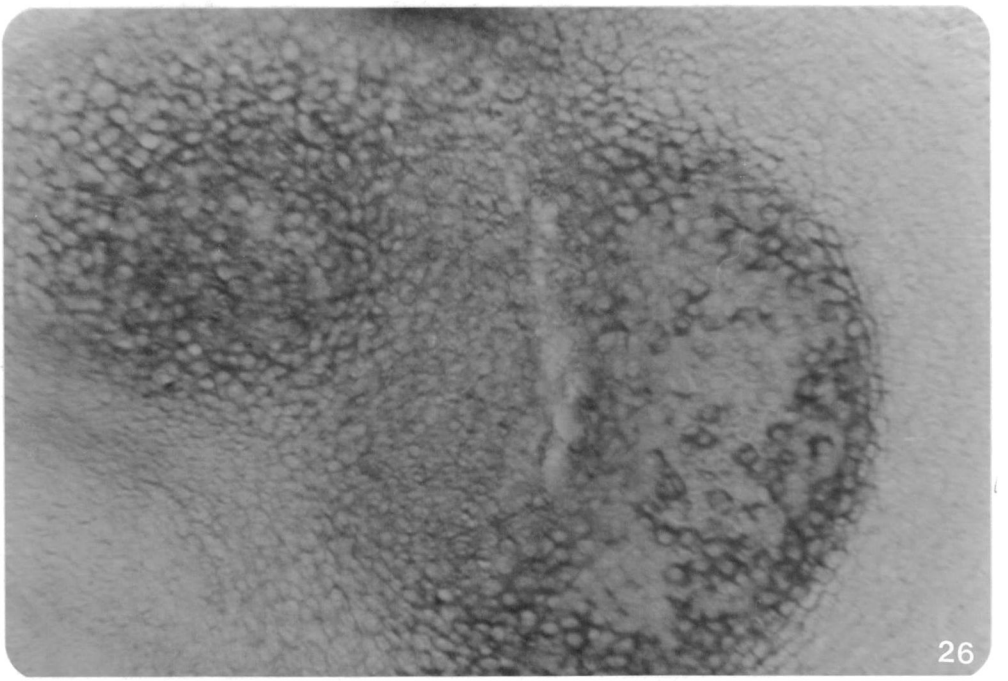
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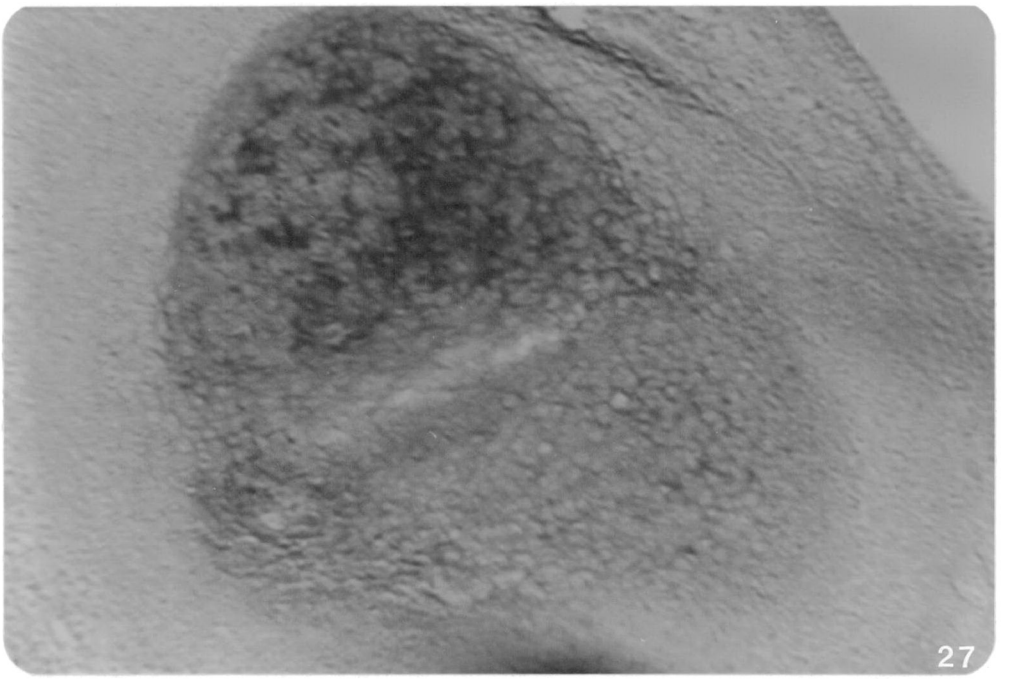
Fig. 27. Longitudinal section of a femural region of 7-day 0.6 cc LACA-treated (2x) embryo (showing matrix is acidophilic and basophilic 10x).

Fig. 28. Longitudinal section of a femoral region of 7-day 0.6 cc LACA-treated (3x) embryo showing acidophilia and basophilia within matrix. Perochondral regions are heavily acidophilic (0x).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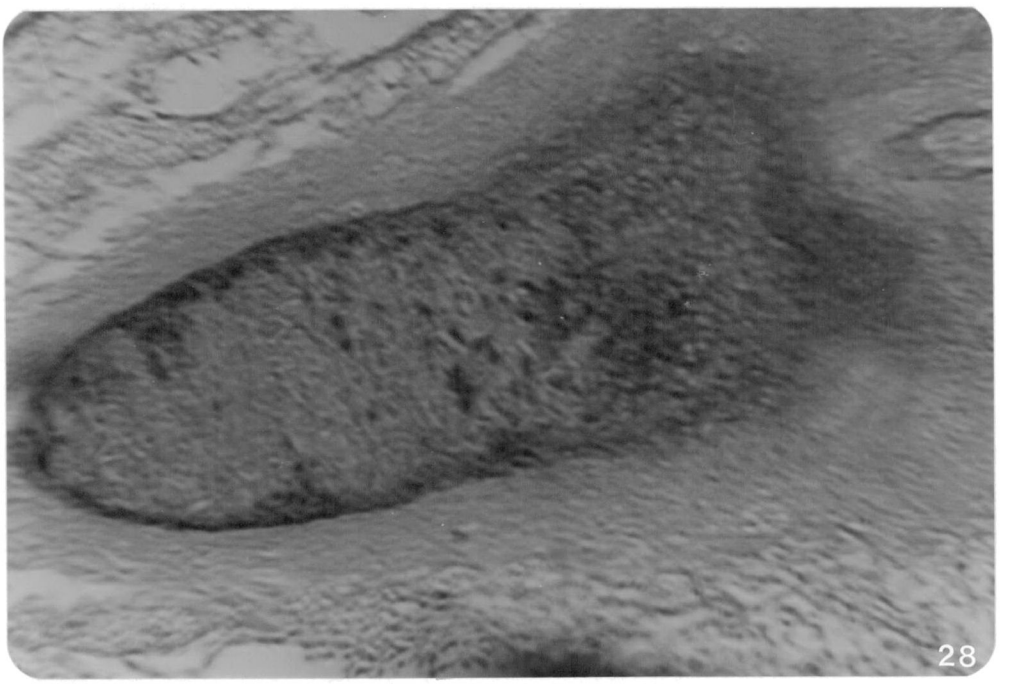
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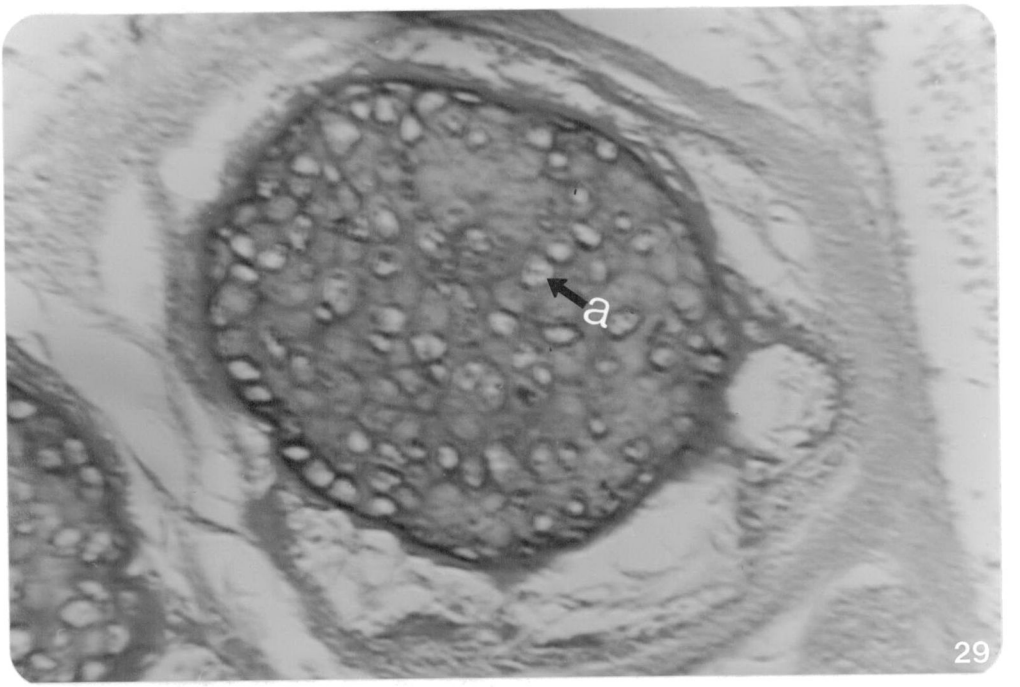
Fig. 29. Cross-section of metatarsus of 9-day control embryo showing chondrocytes (a-arrow), basophilia, and acidophilia in matrix (10x). Perichondral regions are heavily acidophilic, and connective tissue shows acidophilia (10x).

Fig. 30. Cross-section of metatarsus of 9-day 0.6 cc LACA-treated (1x) embryo (showing chondrocytes (b-arrow), basophilia, and acidophilia. Perichondral regions are acidophilic. Connective tissue shows acidophilia 10x).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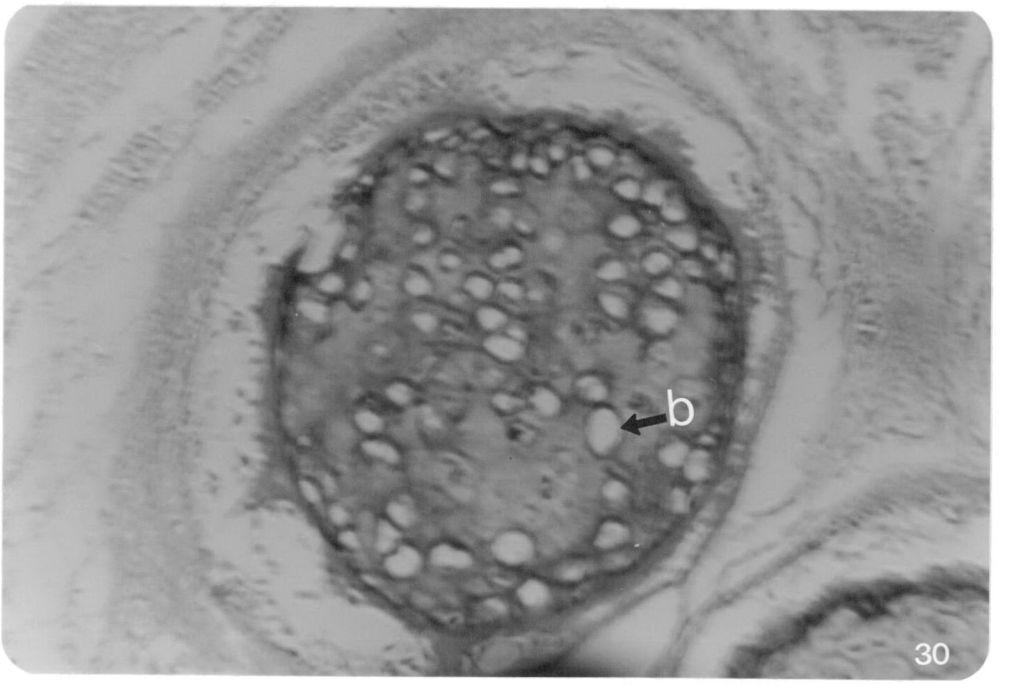
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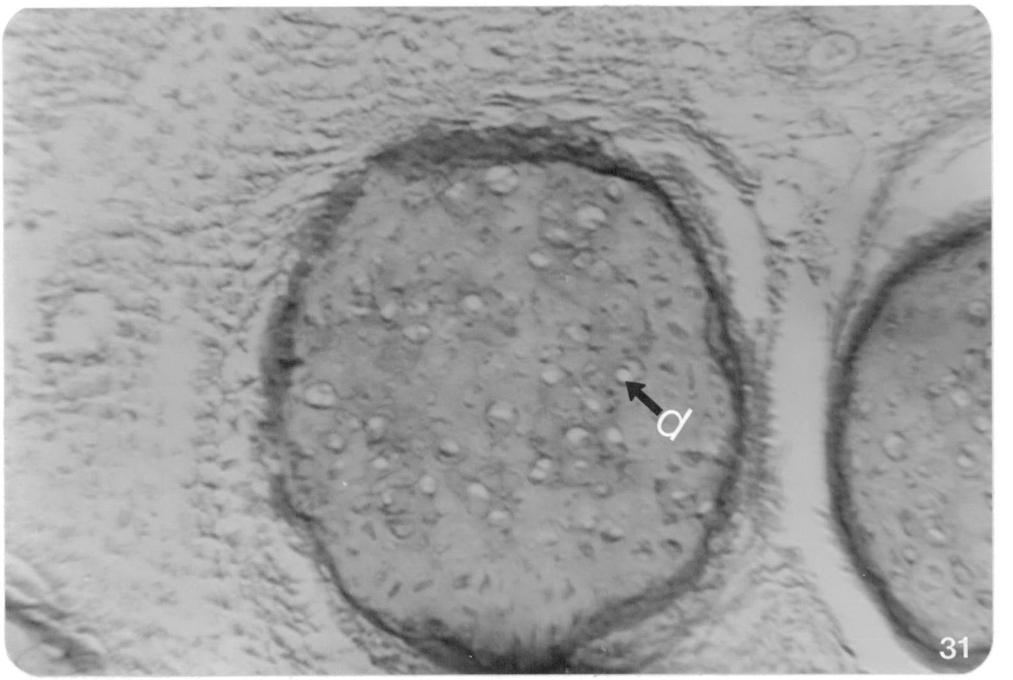
Fig. 31. Cross-section of metatarsus of 9-day 0.6 cc LACA-treated (2x) embryo (showing chondrocytes (d-arrow) basophilia, acidophilia in the matrix. Perichondral regions are acidophilic and connective tissue is lightly acidophilic 10x).

Fig. 32. Cross-section of metatarsus of 9-day LACA-treated (3x) embryo (showing chondrocytes (c-arrow), basophilia, acidophilia. Perichondral regions are acidophilic 10x).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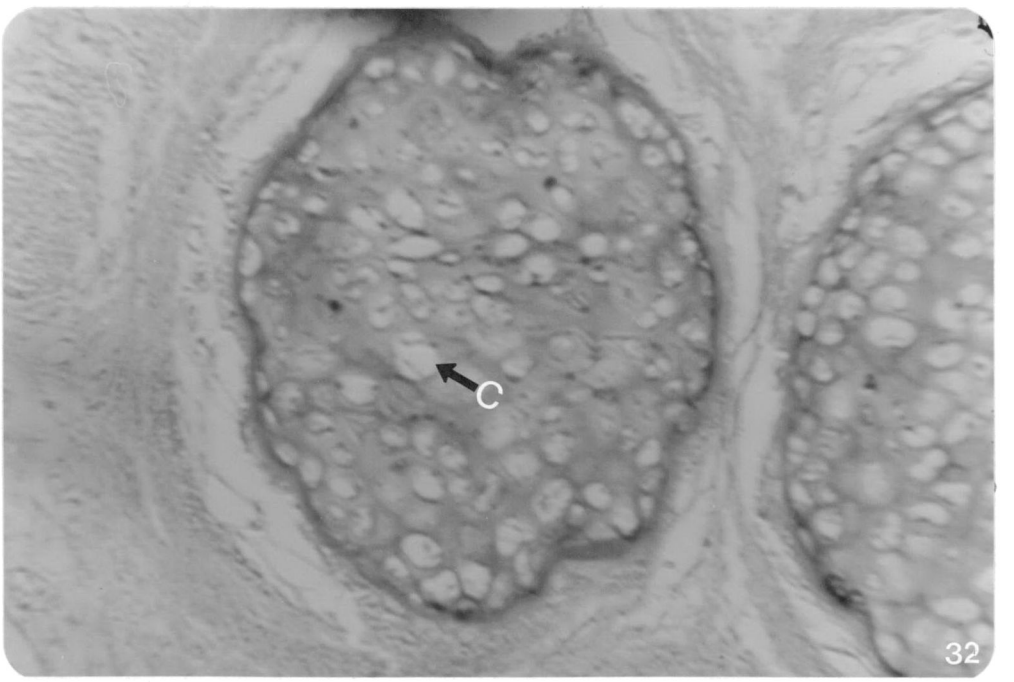
c
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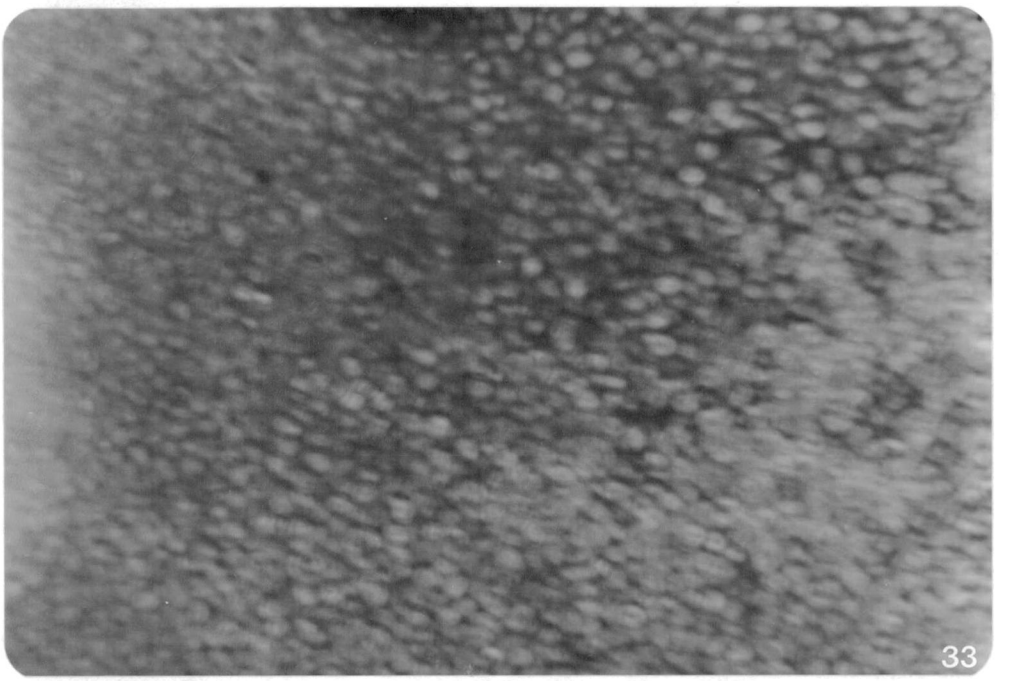
Fig. 33. Sagittal secion of a femoral region of 9-day control embryo (showing basophilia, acidophilia mesenelymal cells 10x).

Fig. 34. Sagittal section of a femoral region of 9-day 0.6 cc LACA-treated (1x) embryo showing basophilia, acidophilia (10x).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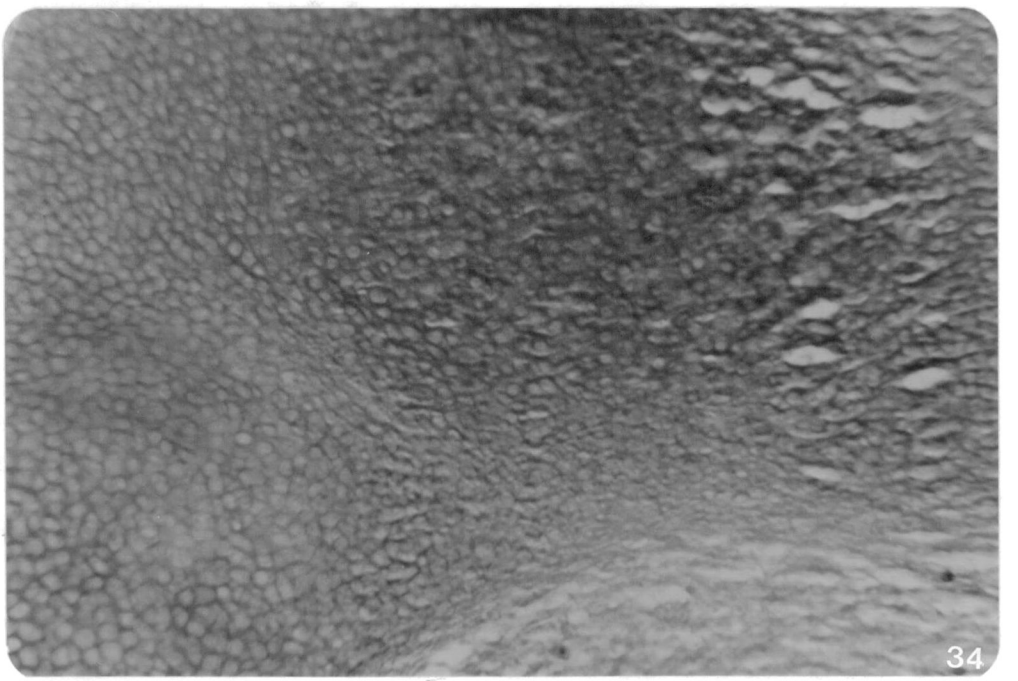
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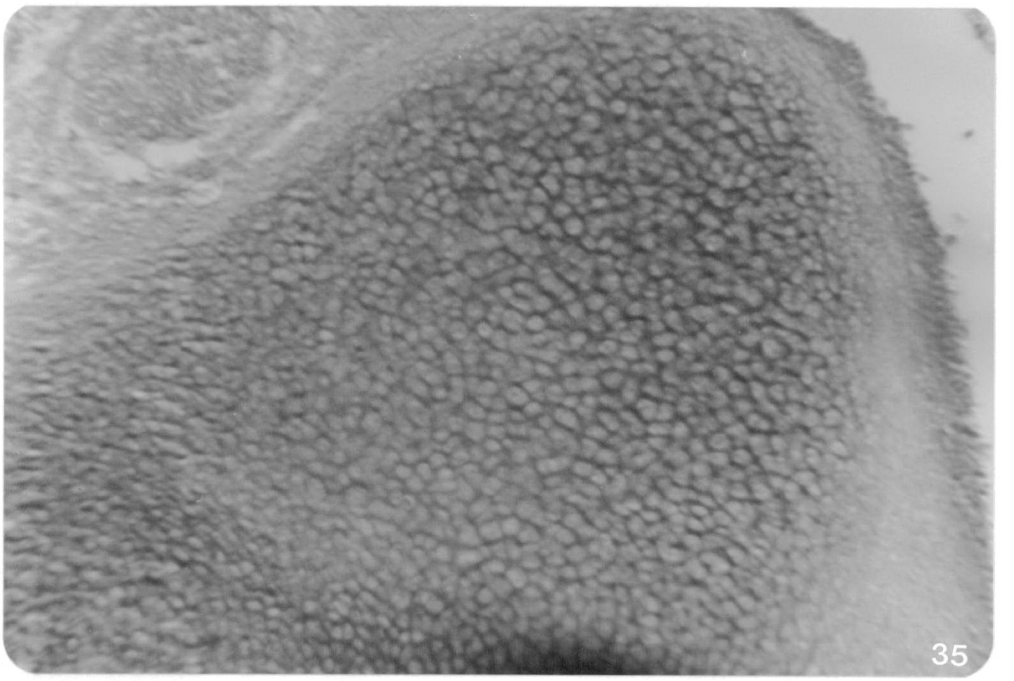
Fig. 35. Sagittal section of a femoral region of 9-day 0.6 cc LACA-treated (2x) embryo (indicating basophilia, acidophilia, mesenehymal cells. Perichondral regions are acidophilic 10x).

Fig. 36. Sagittal section of a femoral region of 9-day 0.6 cc LACA-treated (3x) embryo (showing chondrocytes, basophilia, acidophilia, connective tissue 10x).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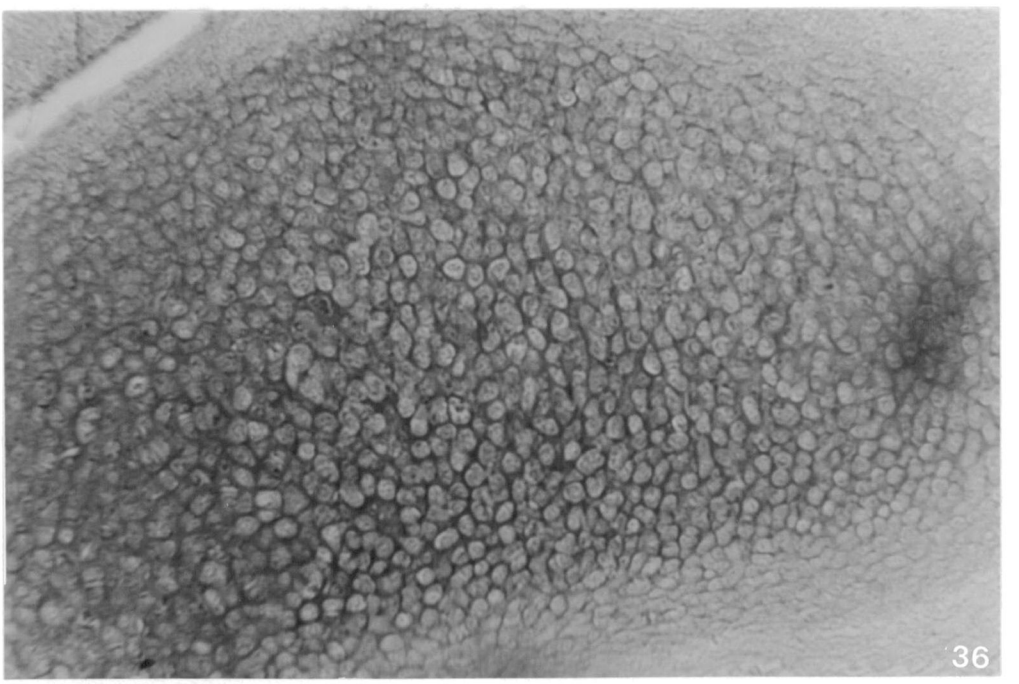
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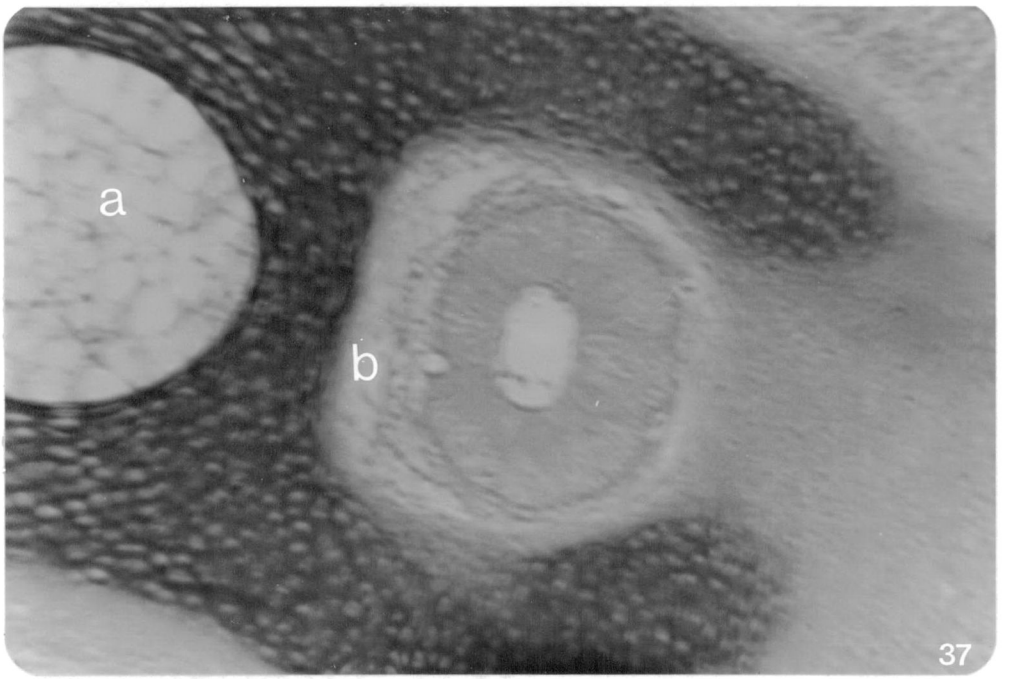
Fig. 37. Cross-section of a vertebral region of 9-day control embryo showing notochord (a), spinal cord (b), basophilia, acidophilia, connective tissue (10x).

Fig. 38. Cross-section of a vertebral region of 9-day 0.6 cc LACA-treated (1x) embryo (showing spinal cord, basophilia, acidophilia, perichondral regions 10x).


a
b
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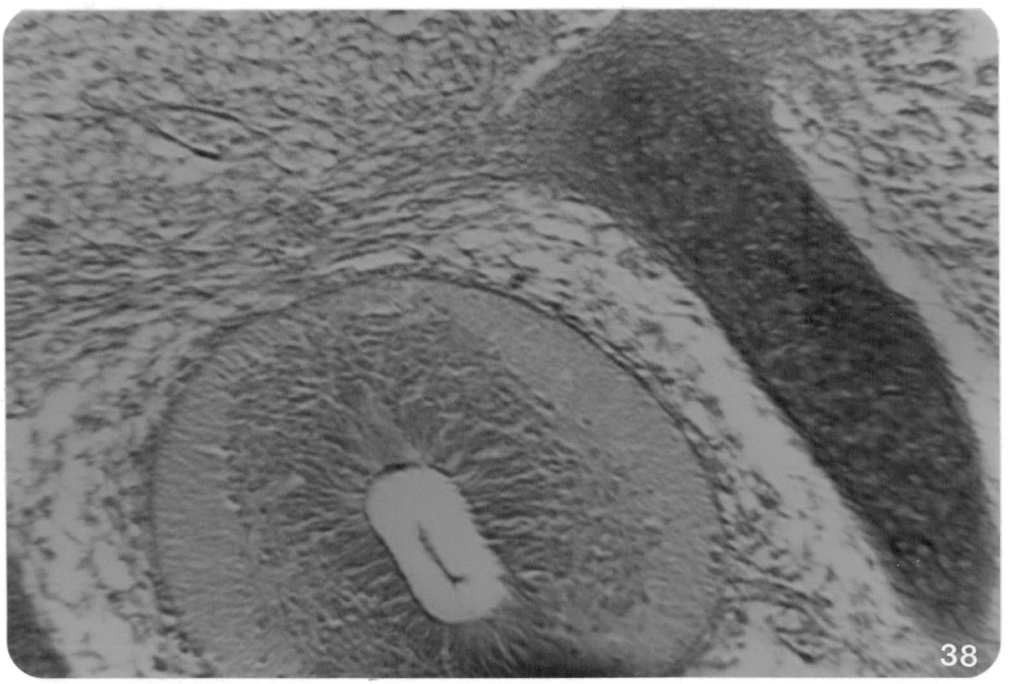
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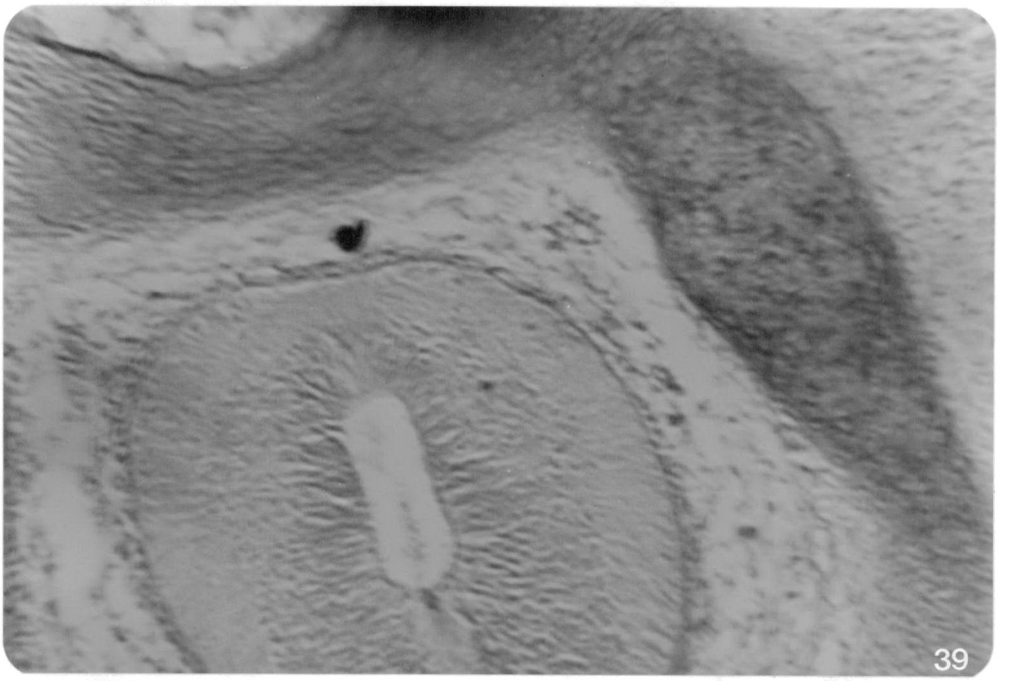
Fig. 39. Cross-section of a vertebral region of 9-day 0.6 cc LACA-treated (2x) embryo (showing spinal cord, basophilia, acidophilia 10x).

Fig. 40. Cross-section of a vertebral region of 9-day 0.6 cc LACA-treated (3x) embryo (showing spinal cord, basophilia, acidophilia, connective tissue 10x).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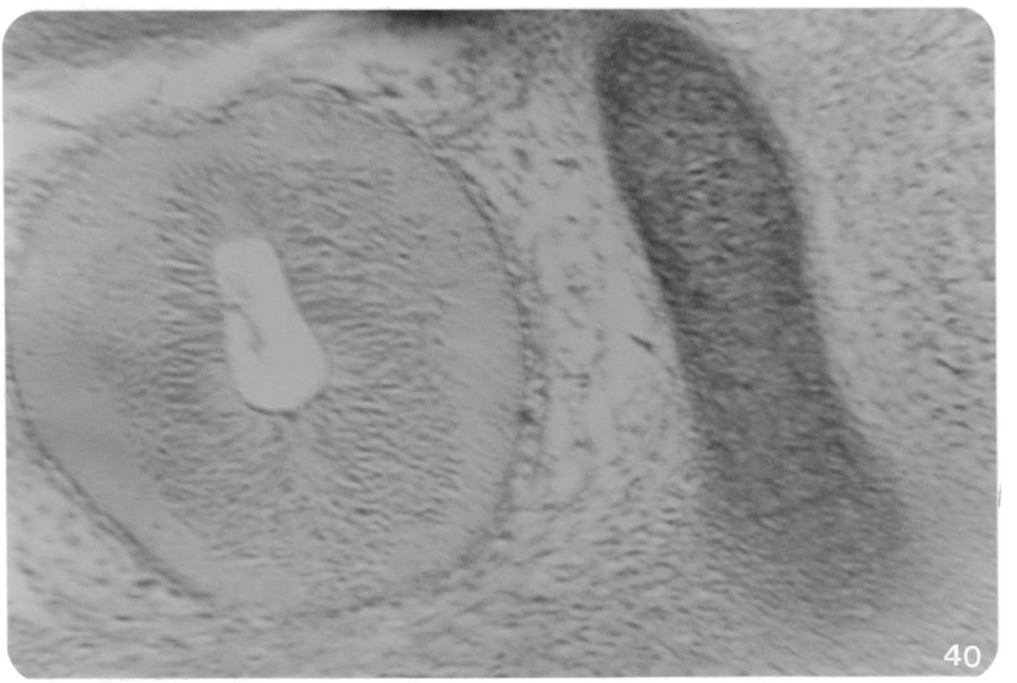
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High Iron Diamine Reactions

The diamine-Alcian blue results for glycosaminoglycans during skeletogenesis are shown in Table 3. The high iron diamine method for sulfated mucosubstances stained the sulfated acid mucopolysaccharides (SM) dark brown and nonsulfated acid mucopolysaccharides blue (Spicer, 1965; Leppi and Spicer, 1967). From this table it was observed that at 7 days and day 9 of control and experimental Group I, the reaction of diamine with sulfated acid mucopolysaccharides (SM) ranged from mild to intense, whereas in experimental Groups II and III the reaction ranged from no reaction to slight reaction. The reaction of Alcian blue with nonsulfated acid mucopolysaccharides (NSM) at day 7 was mild to intense in both control and experimental Group I, but slight to mild in Groups II and III of the same age. At age 9 days, the reaction of Alcian blue with NSM in both control and experimental Group I was intense, but slight to mild in Groups II and III.

Sections through the thighs of 7-day control and experimental Group I embryos showed heavy dark-brown staining in all regions of cartilage (Figs. 41, 42). The stain indicates sulfated acid mucopolysaccharides (SM) (chondroitin sulfate). There was also observed blue-green staining, which was sparsely distributed within the cartilage. This stain indicates nonsulfated acid mucopolysaccharides (NSM). The SM of the control (Fig. 41) was more prominent than that of

Table 3. The effects of L-azetidine-2-carboxylic acid (1 mg/ml) on glycosaminoglycans during skeletogenesis.

Group	# Embryos Injected	Dosage Given	# Injections Given	Diamine and Alcian Blue Indications			
				SM (Days)		NSM (Days)	
				7	9	7	9
Controls (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3*	++/+++	++/+++	++/+++	+++
Experimentals							
Group I (Injected Pre-incubation)	245	0.6 cc	1	++/+++	++/+++	++/+++	+++
Group II (Injected Pre-incubation, 48 hr)	245	0.6 cc	2	+/++		+/++	+/++
Group III (Injected Pre-incubation, 48 hr, 96 hr)	245	0.6 cc	3	0/+	0/+	+/++	+/++

+++ Intense Reaction; ++ Mild Reaction; + Slight Reaction; 0 No Reaction.

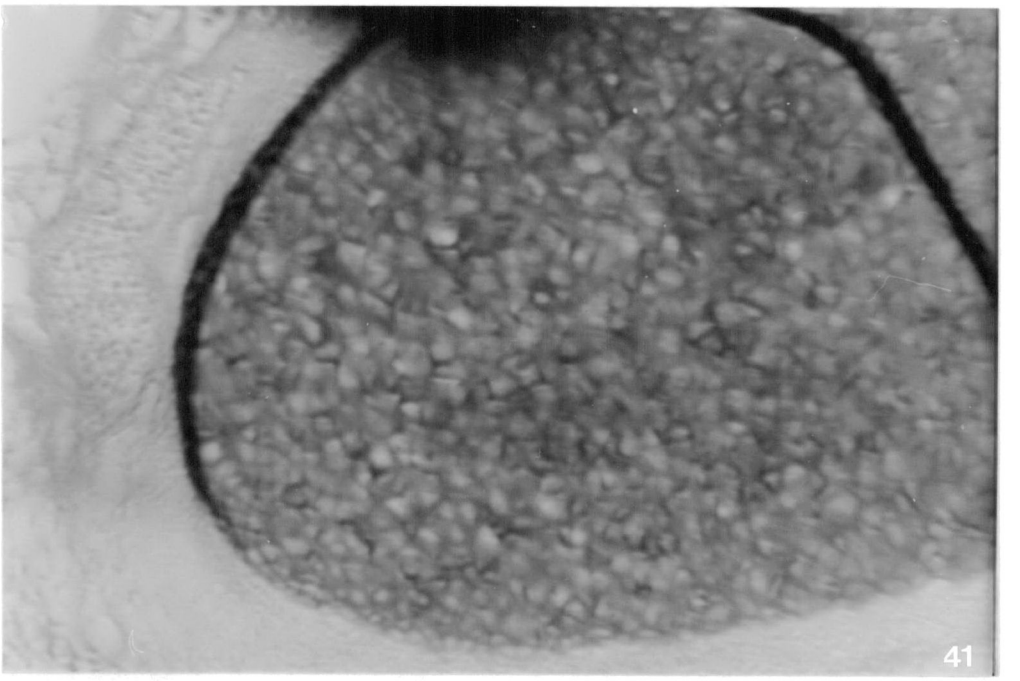
*Controls were injected pre-incubation, pre-incubation plus 48 hr, pre-incubation, 48 hr, and 96 hr. Since no differences were noted, the triply injected are cited here.

Group I (Fig. 42). Examination of Groups II and III (Figs. 43, 44) revealed a reversal in staining to that of control and Group I (Figs. 41, 42). In these groups (II, III) the NSM was more heavily distributed throughout the cartilage than the SM. The SM was mildly stained and sparsely distributed.

Cross-sections through the vertebral region of 9-day embryos were observed for the analysis of hyaluronic and chondroitin acids. Control and experimental Group I showed a preponderance of SM in the cartilage of the neural arch of the skeleton (Figs. 45, 46). The NSM in these groups was mild and sparsely distributed within the matrix of the cartilage. But in the perichondral regions the NSM was intense and hardly any SM was observed. In experimental Group II, the cartilage within the neural arch showed heavy sites of NSM and a low level of SM (Fig. 47). The region outside the neural arch of the skeleton also showed NSM; however, the experimental Group III (Fig. 48), though showing NSM to be predominant, is less than that of experimental Group II, and the SM in this group was much less than that of Group II. Both of these groups showed NSM in the perichondral regions. The notochordal regions showed NSM levels.

Fig. 41. Sagittal section of a femoral region of 7-day control embryo (showing sulfated mucosubstances (dark-brown), nonsulfated mucosubstances (blue-green).

Fig. 42. Sagittal section of a femoral region of 7-day 0.6 cc LACA-treated (1x) embryo (showing sulfated mucosubstances (dark-brown) and nonsulfated mucosubstances (blue-green) within cartilage 10x).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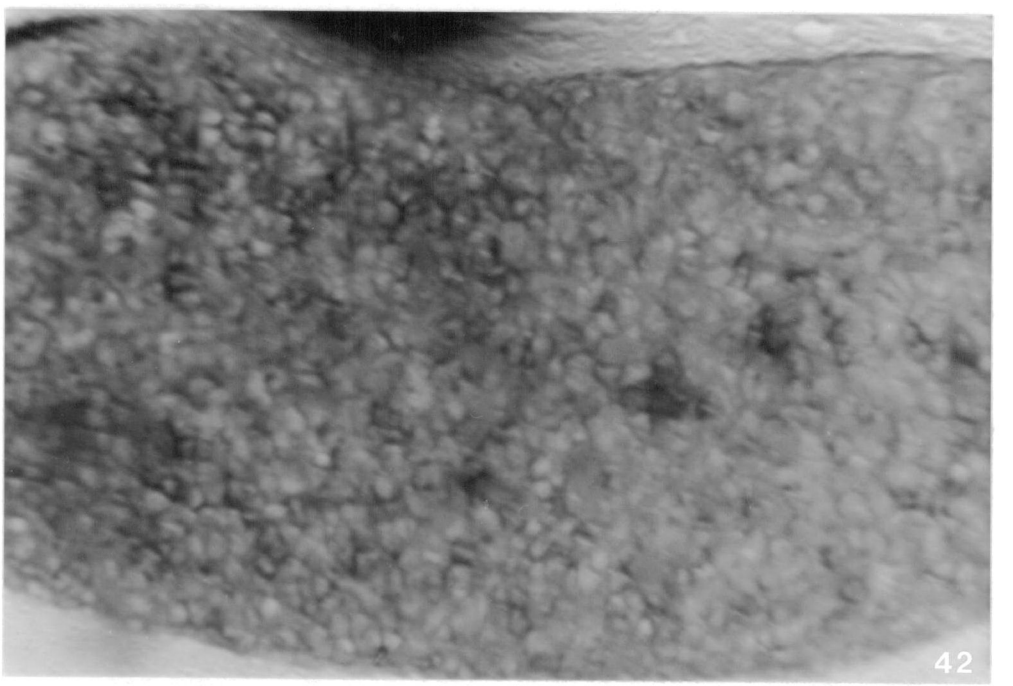
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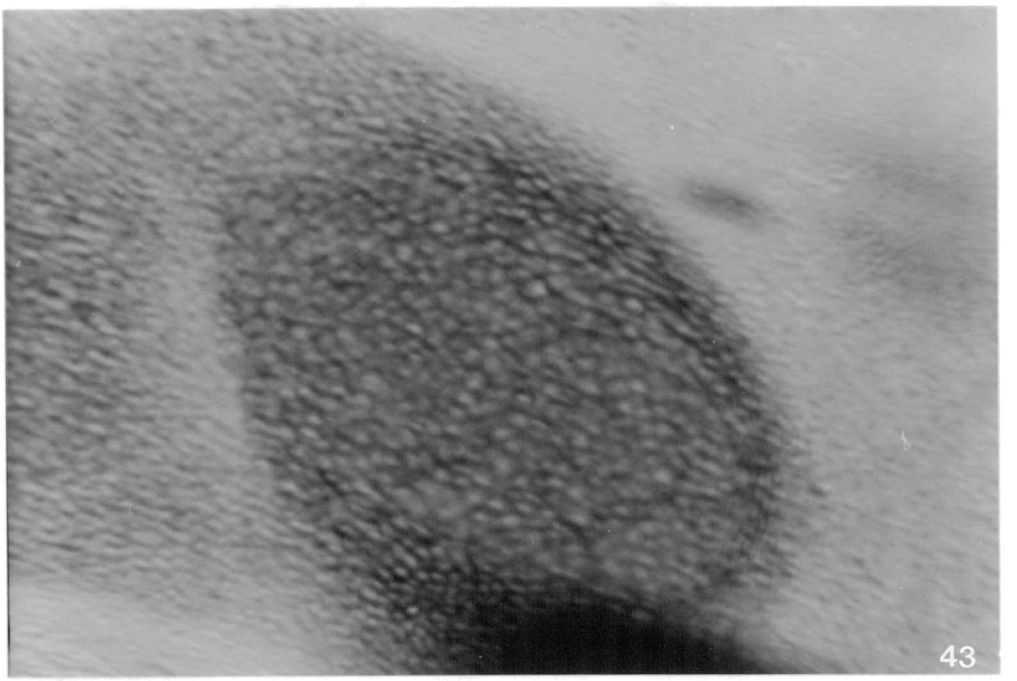
Fig. 43. Sagittal section of a femoral region of 7-day 0.6 cc LACA-treated (2x) embryo (showing sulfated mucosubstances and nonsulfated mucosubstances 10x).

Fig. 44. Sagittal section of a femoral region of 7-day 0.6 cc LACA-treated (1x) embryo (showing sulfated mucosubstances and nonsulfated mucosubstances 10x).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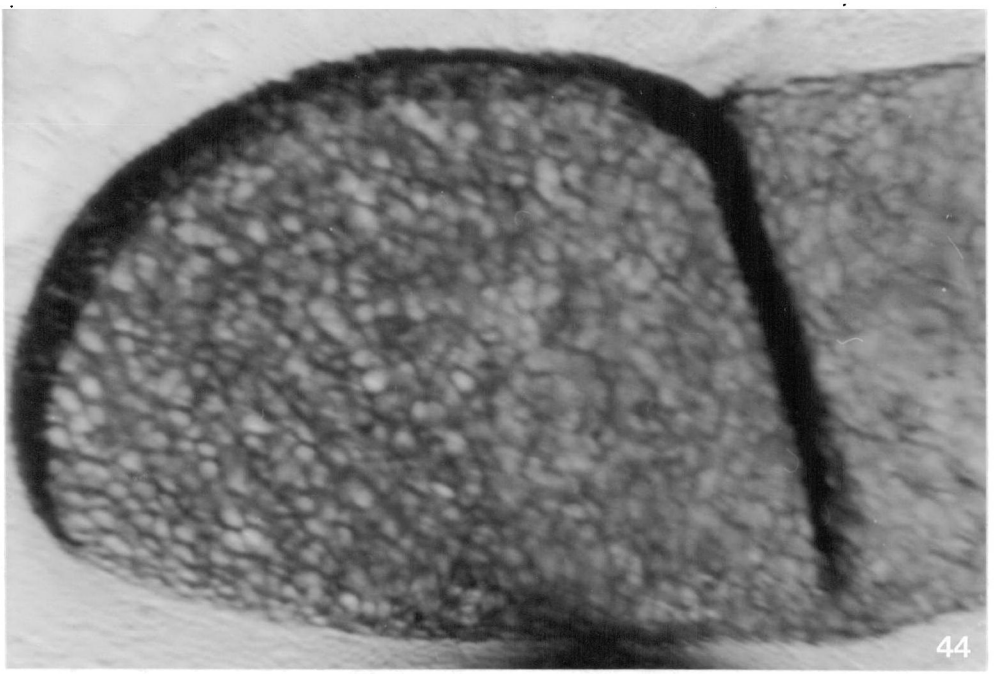
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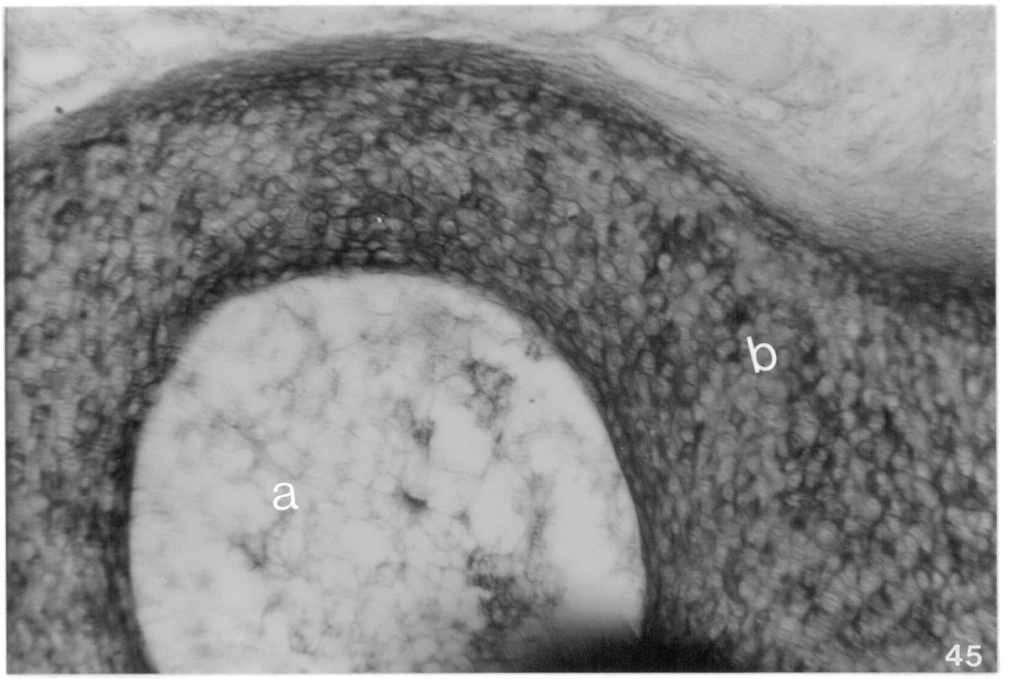
Fig. 45. Cross-section of vertebral region of 9-day control embryo (showing notochord (a), centrum formation (b). Dark-brown within cartilage shows sulfated mucosubstances, blue-green shows nonsulfated mucosubstances. Perichondral regions show nonsulfated mucosubstances 10x).

Fig. 46. Cross-section of vertebral region of 9-day 0.6 cc LACA-treated (1x) embryo (showing spinal canal (a), gray matter (b), white matter (c), neural arch of skeleton (d). Cartilage shows dark-brown (acidophilia), and basophilia (blue-green). Perichondral region - basophilic 10x).


a
b
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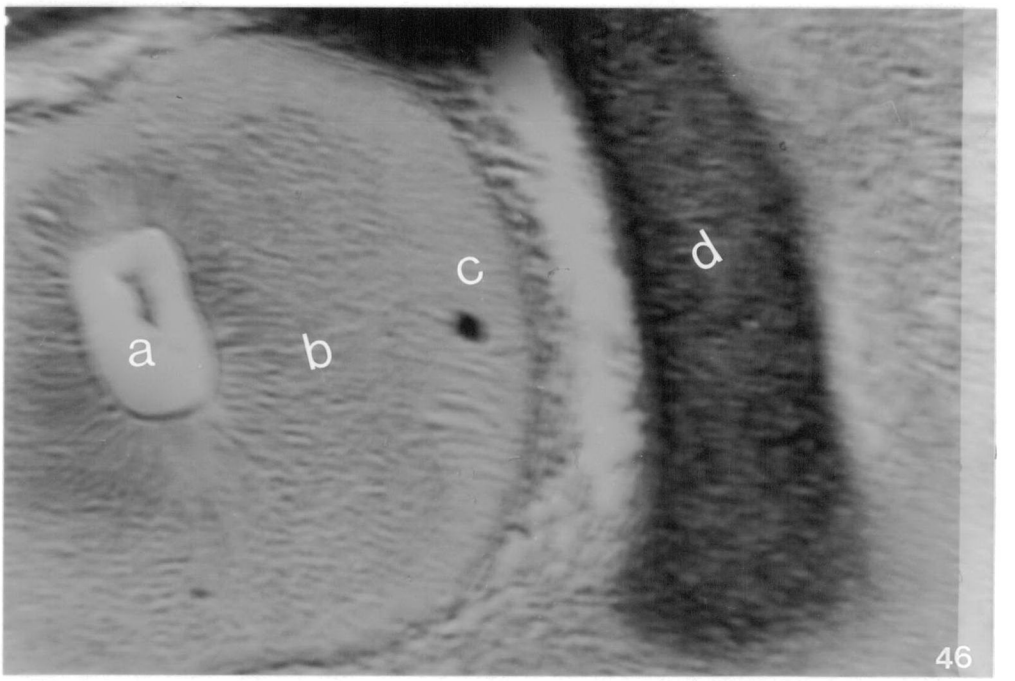
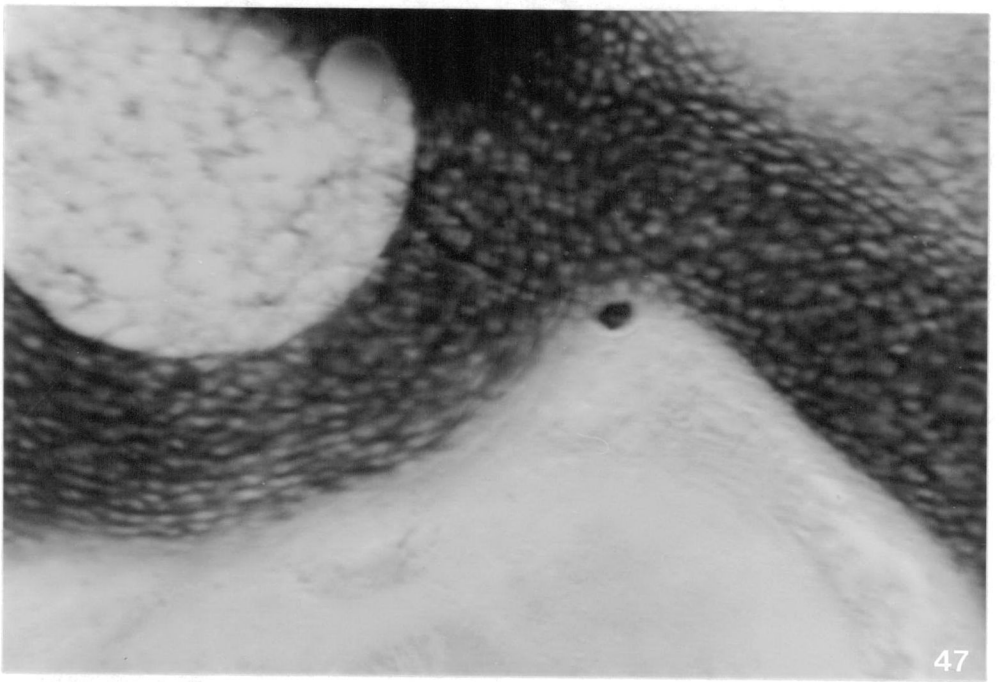

a
b
c
d
46

Fig. 47. Cross-section of vertebral region of 9-day 0.6 cc LACA-treated (2x) embryo (showing nonsulfated mucosubstances (blue-green), sulfated mucosubstances (dark-brown) 10x).

Fig. 48. Cross-section of vertebral region of 9-day 0.6 cc LACA-treated (3x) embryo (showing notochord, and vertebra. Note the sulfated mucosubstances (dark-brown) and the non-sulfated mucosubstances (blue-green) 10x).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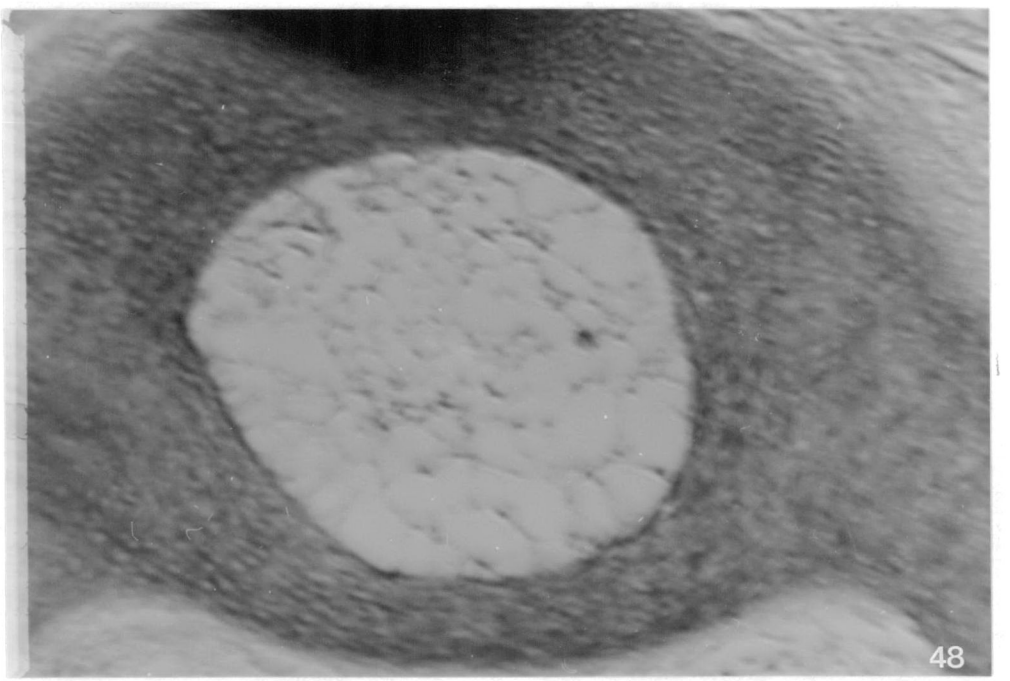
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CHAPTER V

DISCUSSION

L-azetidine-2-carboxylic acid (LACA) has been used widely by numerous investigators to study its effects on collagen synthesis and subsequent release into the extracellular environment. Its effects on growth phenomena have also been noted. The current studies point to the inhibition of calcium deposition in chicken embryos resulting from the exposure of such embryos to LACA. Too, histochemical studies of Alcian blue-PAS staining and diamine method for glycosaminoglycans indicate some possible involvement of this analog of proline on the matrix of collagen during skeletogenesis.

Aydelotte and Kochhar (1972) demonstrated that LACA adversely affected collagen synthesis and caused alterations in limb-bud morphogenesis and skeletal rigidity. Findings of the present in vivo study are supportive of their data based on in vitro observations. Several of the LACA-treated embryos showed inhibition of calcium deposition in their skeleton. As was pointed out by Bhagavan (1978), calcium is the most important cation in the skeleton and 99% of it is found in the skeleton. The other is found in interstitial, extravascular, and intravascular components of the body. The calcium existing in these parts of the body is calcium

chloride. In animal tissues, the types of calcium salts normally found include calcium carbonate, calcium oxalate, and calcium phosphate. Alizarin Red S has been found not to stain oxalate salts (Brown, 1978). In the treated 7-day old embryos, especially those receiving 2 or 3 injections, the phalanges, metacarpalia, and the cranio-vertebral regions showed no calcium deposition. In embryos receiving 3 injections (Group III), the skeletal regions where the calcium deposition was observed, was only patchy. The investigation of O'Dell (1966) showed that LACA caused limb bone rudiments and the mandibular cartilage to become bent and very soft, thus supporting the findings here which showed inhibition of calcium deposition.

There is ample documentation that calcium deficiency leads to osteoporosis in the elderly or rickets in children (Bhagavan, 1978). The experimental embryos (especially Groups II and III) of the present study showed very little calcium deposition in their cranio-vertebral regions, suggesting that skeletogenesis was retarded. This finding was in agreement with the findings of Sudo et al. (1977) that LACA caused skeletal abnormalities in the lumbar and sacral vertebrae in mice. Additionally, Nagai et al. (1976) showed that LACA caused malformation in the fetuses of rats and a reduction in ossification of the sternebrae. Brian (1978) reported that the secondary chondrogenesis of the quadratojugal, a membrane bone of the skull, was inhibited by treatment

of the whole embryos with LACA. In several of the experimental embryos observed here, calcium failed to be deposited in the cranial regions.

Strudel (1975) showed that LACA inhibited the secretion of the periaxial extracellular material, resulting in a lack of several vertebrae. In young vertebral primordia cultured in LACA, the myotome cells differentiated into myoblasts, whereas the sclerotome cells remained undifferentiated. In older primordia, the sclerotome cells gave rise to abnormal cartilage but the myotome cells seldom differentiated. Current observations indicated that most of the extracellular material of the experimental embryos, especially Groups II and III, showed acidophilia with few areas in the cartilage showing basophilia. The areas in the cartilage and perichondral regions showing acidophilia indicated the presence of neutral acid mucopolysaccharides, and those areas showing basophilia, acid mycopolysaccharides.

As pointed out earlier, acid mycopolysaccharides are divided into two categories--nonsulfated acid mycopolysaccharides (hyaluronic acid) and sulfated acid mucopolysaccharides (chondroitin sulfate). Hyaluronic acid is present in connective tissue, whereas chondroitin sulfate A and C are major structural components of bone and cartilage. In the present studies, sections of the control and experimental Group I showed heavy deposition of calcium at the perichondral regions, whereas little or none was observed in the

cartilage. In the embryos of Groups II and III, calcium deposition was observed to range from mild to zero. These suggested that in the presence of LACA calcium deposition was inhibited, causing retardation of skeletogenesis. This was supported by the observations of Aydelotte (1971) when he showed that LACA interfered with normal development of cartilage, after culturing the mouse limb-buds for 9 days in the presence of LACA. When the sections were stained for Alcian blue-PAS reaction, perichondral regions of both the control and the experimental embryos showed acidophilia, indicating neutral acid mucopolysaccharides. The matrix of both the control and experimental Group I showed extensive basophilia and less acidophilia, indicating the production of both neutral and acid mucopolysaccharides. However, in experimental Groups II and III, very little basophilia was observed in the matrix, suggesting that LACA had an effect on the synthesis of acid mucopolysaccharides. These findings were in agreement with those of Johnson et al. (1973), who showed that the cardiac jelly, the extracellular matrix of the developing chick heart, consists mainly of glycosanimoglycans. They observed in vitro that in the presence of LACA, myocardial cytodifferentiation and normal cardiac morphogenesis were inhibited markedly.

From current studies so far the assumption can be made that even though calcification was affected by LACA, the synthesis of neutral acid mucopolysaccharides was not affected.

To further test this assumption, sections of the control and the experimental embryos were treated in diamine solution to analyze nonsulfated and sulfated acid mucopolysaccharides. The control and the experimental Group I showed intense dark-brown staining, an indication of sulfated acid mucopolysaccharides in the matrix, whereas nonsulfated acid mucopolysaccharides (showing blue-green staining) though present seemed to be a bit less than sulfated acid mucopolysaccharides. In the experimental Groups II and III, there was more blue-green staining observed than dark-brown staining. Earlier, it was pointed out that in the diamine reaction, the blue-green indicates hyaluronic acid while the dark-brown indicates chondroitin acid. Therefore, in the experimental Groups II and III, which showed more nonsulfated acid mucopolysaccharides than sulfated acid mucopolysaccharides, LACA in some ways inhibited the synthesis of sulfated acid mucopolysaccharides--the major component of cartilage and bone.

In their study Coulombre and Coulombre (1972) demonstrated that administration of LACA to growing chick embryos caused the Golgi apparatus not to stain with silver. This supported our findings from the technique using Alcian blue-PAS that the acid mucopolysaccharides were inhibited, and from the diamine procedure that the sulfated acid mucopolysaccharides were inhibited. The failureof Golgi apparatus to stain with silver, as was demonstrated by Coulombre and

Coulombre, showed that LACA interfered with the matrix. The failure of the matrix to show basophilia in Alcian blue-PAS method and dark-brown in diamine method further substantiated this work on the Golgi apparatus.

When the embryos were treated in Alizarin Red S, the control and experimental Group I showed heavy calcification in the perichondral regions which was supported by the observations of Holder (1978). In his studies of Alizarin Red S, Holder demonstrated the onset of osteogenesis in the developing chick limb from 7 days to 19 days. He also demonstrated the chronological order of appearance of ossification centers in the skeletal elements of the developing wing, and pointed out that the ossification of the phalanges begins sooner in the leg than in the wing. Contrarily, experimental Groups II and III showed mild to zero calcification in their perichondral regions. Treatment of the sections from control and experimental embryos I, II, and III raised a question as to what types of glycosaminoglycans are preponderant in the perichondral regions of the cartilage of a chick embryo. The possible response to this may be that since it has been shown that mucosubstances contain hexosamine sugars in combination with different amounts of proteins and some lipids (Bancroft, 1975), it is likely that in the perichondral regions of the cartilage of a chick embryo the preponderant molecules are neutral acid mucopolysaccharides, which are PAS positive and react similarly to muco-proteins. However,

the exact role of neutral acid mucopolysaccharides in skeletogenesis has not yet been determined.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Injections of L-azetidine-2-carboxylic acid (LACA), 0.6 cc of 1 mg/ml, were given to experimental embryos Group I once (pre-incubation), Group II twice (pre-incubation and 48 hr), and Group III thrice (pre-incubation, 48 hr, and 96 hr).

2. The limb-buds of 7-day-old embryos showed little or no calcification. In 9-day-old embryos, though there was visible calcification in most of the regions of the limb-buds and the vertebrae, embryos of Groups II and III showed less calcification.

3. Sections through the limb-buds and vertebral regions showed mild to no calcification in some regions of perichondrium of the cartilage.

4. Sections treated in Alcian blue-PAS showed less sulfated acid mucopolysaccharides and heavy nonsulfated acid mucopolysaccharides within the cartilage and at the perichondrial regions.

5. Sections treated in diamine solution also showed less sulfated acid mucopolysaccharides and heavy nonsulfated acid mucopolysaccharides within the matrix of the cartilage.

6. From this study the conclusion is warranted that LACA affects calcification and inhibits the synthesis of

acid mucopolysaccharides. However, the mechanisms undertaken to cause the effects and the inhibition in chick embryos will have to await further investigations.

LITERATURE CITED

Alescio, T. 1973. Effect of a proline analogue, azetidine-2-carboxylic acid, on the morphogenesis in vitro of mouse embryonic lung. J. Embryol. Exp. Morphol. 29: 439-451.

Aydelotte, M. B. 1971. Effects of azetidine-2-carboxylic acid on developing chick limb buds in vitro. Anat. Rec. 168: 281-305.

_____ and D. M. Kochhar. 1972. Influence of L-azetidine-2-carboxylic acid on developing mouse limb in vitro. Anat. Rec. 169: 269-270.

Baich, A., and F. J. Smith. 1968. The effect of azetidine-2-carboxylic acid on the synthesis of proline in Escherichia coli. Experientia 24: 1107.

Bancroft, J. D. 1975. Histochemical Techniques. Butterworths. London and Boston. Chapter 7. Carbohydrates (pp. 69).

Barzansky, B. M. 1974. Effect of the proline analogue azetidine-2-carboxylic acid on head regeneration in Hydra. Anat. Rec. 178: 358.

_____. 1975 Production of an abnormal nematocyst morphology in Hydra. Anat. Rec. 181: 308.

Baum, B. J., R. F. Troxler, H. M. Kagan, J. A. Grasso, B. Faris and C. Franzblau. 1973. Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit

reticulocytes. Biochim. Biophys. Res. Commun. 53: 1350-1356.

Bhagaran, N. V. 1978. Biochemistry. J. B. Lippincott Company. Philadelphia. Chapter 9, Mineral Metabolism (pp. 980).

Brown, G. G. 1978. An Introduction to Histochemistry. Appleton-Century-Crofts. New York. Chapter 15 (pp. 263).

Cooper, G. W. and D. J. Prockop. 1969. L-azetidine-2-carboxylic acid and the extrusion of collagen. J. Cell. Biol. 38: 523-536.

Coulombre, A. M. and J. L. Coulombre. 1971. Lesions produced in the primary corneal stroma of chick embryos by L-azetidine-2-carboxylic acid. Anat. Rec. 169: 301-302.

_____. 1972. Interruption of collagen excretion into the primary stroma of the cornea with L-azetidine-2-carboxylic acid. Dev. Biol. 28: 182-199.

Dancewicz, A. M. and K. I. Altman. 1967. The effect of azetidine-2-carboxylic acid on in vitro synthesis of collagen in polyvinyl-sponge granulomas from rats. Proc. Soc. Exp. Biol. 26: 832-834.

Dehm, P. and D. J. Prockop. 1971. Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon. Biochim. Biophys. Acta 240: 358-369.

Dunn, M. A., M. Rojkind, K. S. Warren, P. K. Hait, L. Rifas

and S. Selfter. 1977. Liver collagen synthesis in murine schistosomiasis. J. Clin. Invest. 59: 666-674.

Finerman, G. M. and Rosenbert. 1967. Regulation of collagen synthesis by perturbation proline transport. Biochim. Biophys. Acta 135: 1008-1015.

Fowden, L. 1956. Azetidine-2-carboxylic acid: A new cyclic amino acid occurring in plants. Biochim. Biophys. Acta 64: 323-331.

_____. 1963. Replacement of proline by azetidine-2-carboxylic acid during biosynthesis of proline. Biochim. Biophys. Acta 71: 459-461.

Frederickson, R. G. 1974. Effect of proline analogue on the fine structure of perinoto-chordal connective tissue elements in young chick embryos. Anat. Rec. 178: 358.

Grant, M. M., A. S. Brown, L. M. Corwin, R. F. Troxler and C. Franzblau. 1975. Effect of L-azetidine-2-carboxylic on growth and proline metabolism in Escherichia coli. Biochim. Biophys. Acta 404: 180-187.

Hall, B. K. 1978. Use of the L-proline analog, L-azetidine-2-carboxylic acid (LACA), to analyze embryonic growth and determination and expression of the chondrogenic phenotype in vivo and in vitro. Anat. Rec. 190: 243-256.

Hilfer, S. R. and G. L. Pakstis. 1977. Interference with thyroid histogenesis by inhibitors of collagen synthesis. J. Cell. Biol. 75: 446-463.

Holder, N. 1978. The onset of osteogenesis in the developing chick limb. J. Embryol. Exp. Morph. 44: 15-29.

Hollister, G. 1934. Clearing and dyeing fish for bone study. Zoologica 12: 89-101.

Hood, R. C., W. S. and W. M. Neill. 1948. A modification of Alizarin Red S technic for demonstrating bone formation. Stain Technology 23: 209-218.

Johnson, R. C., F. J. Manasek, W. C. Vinson, and J. M. Seyer. 1973. Collagen in early cardiogenesis. Anat. Rec. 175: 350-351.

Kambarask, K., H. Sudo, A. Hiura, H. Nagai and N. Nagai. 1976. Effects of L-azetidine-2-carboxylic acid upon the incisor in mice and rats. Teratol. 14: 242.

Kershenobich, D. and M. Rojkind. 1973. Effect of the administration of L-azetidine-2-carboxylic acid on albumin and transferring biosynthesis by liver slices of rats treated with carbon tetrachloride. Biochim. Biophys. Acta 319: 216-222.

Koch, W. E. 1974. The effects of azetidine-2-carboxylic acid on cultured incisors of embryonic mice. Anat. Rec. 178: 393-394.

Krochmal, A., R. S. Wlaters, R. M. Doughty. 1971. A guide to medicinal plants of Appalachia (pp. 1, 40, 82, 202, 256, 262).

Lampe, K. F. 1974. Systematic plant poisoning in children. Pediat. 54: 347-351.

Lane, J. M., P. Dehm and D. J. Prockop. 1971a. Proline analogue azetidine-2-carboxylic acid on collagen synthesis in vivo. I. Arrest of collagen accumulation in growing chick embryos. Biochim. Biophys. Acta 236: 517-527.

_____, L. J. Parkes, and D. J. Prokop. 1971b. Effect of proline analogue azetidien-2-carboxylic acid on collagen synthesis in vivo. II. Morphological and physical properties of collagen containing the analogue. Biochim. Biophys. Acta 236: 528-541.

_____, D. J. Prockop, P. Dehm and L. J. Parkes. 1971. Inhibition of collagen synthesis in chick embryos by the proline analogue L-azetidine-2-carboxylic acid. J. Bone Surg. Am. 53: 1018.

Leppi, J. J. and S. S. Spicer. 1967. High iron diamine method for sulphated mucosubstances. Anat. Rec. 159: 179.

Leushner, J. and J. Pasternak. 1978. Collagen production by the free-living nematode Panagrellus silusiae in the presence of the proline analogue L-azetidien-2-carboxylic acid. J. Exp. Zool. 204: 155-162.

Lewis, W. H. 1977. Medical Botany. John Wiley and Sons, New York.

Mowry, R. W. 1956. Alcian blue-PAS method. J. Histochem. Cytochem. 4: 407.

Nagai, H., S. Yokoyama, K. Kambara, H. Sudo, T. Katsuya and

N. Nagai. 1976. Effects of L-azetidine-2-carboxylic acid upon the rat fetus. Teratol. 14: 248.

O'Dell, D. S. 1966. The arrest of the accumulation of collagen in the tissue by L-azetidine-2-carboxylic acid. Exp. Cell Res. 40: 432-438.

Oikarinen, A., H. Antlinen and K. I. Kivirikko. 1976. Effect of L-azetidine-2-carboxylic acid on glycosylations of collagen in chick embryo tendon cells. Biochem. J. 160: 639-645.

Pitman, M. G., R. A. Wildes, W. Schaffer and D. Wellfare. 1977. Effect of azetidine-2-carboxylic acid on ion uptake and ion release to the xylem of excised barley roots. Plant Physiol. 60: 240-246.

Reynolds, J. J. 1965. The effect of L-azetidine-2-carboxylic acid on the growth of chick bone rudiments. Exp. Cell Res. 42: 178-197.

Rojkind. J. 1973. Inhibition of liver fibrosis by L-azetidine-2-carboxylic acid in rats treated with carbon tetrachloride. J. Clin. Invest. 52: 2451-2456.

_____ and D. Kershenobich. 1975. Effects of colchicine on collagen, albumin, and transferrin synthesis by cirrhotic rat liver slices. Biochim. Biophys. Acta 378: 415-423.

Ruch, J. V., M. Fabre, V. Karsher-Djuricic and A. Stäubli. 1974. The effects of L-azetidine-2-carboxylic acid (analogue of proline) on dental cytodifferentiation

in vitro. Different. 2: 211-220.

Satir, B. 1975. The final steps in secretion. Sci. Am. 233: 29-37.

Schneider, A. M. and R. M. Kuff. 1954. Chondroitin sulfate and collagen in inherited skeletal defects of chickens. Exp. Cell Res. 31: 325-346.

Spicer, S. S. 1965. High iron diamine method for sulphated mucosubstances. J. Histochem. Cytochem. 13: 211.

Strudel, G. 1975. Effects of L-azetidine-2-carboxylic acid and of 5-bromodeoxyuridine in vivo and in vitro on the development of the chick skeleton. Teratol. 12: 336.

Sudo, H., K. Kambara, S. Hokoyama, T. Katsuya, H. Nagai and N. Nagai. 1977. Effects of L-azetidine-2-carboxylic acid on the mouse skeleton and tooth germ. Teratol. 16: 123.

_____, K. Kambara, T. Katsuya, H. Nagai, and N. Nagai. 1978. Disturbance of rat incisor dentine formation following administration of L-azetidine-2-carboxylic acid. Teratol. 18: 148.

Sung, M. L. and L. Fowden. 1969. Azetidine-2-carboxylic acid from legume Delonix regia. Phytochem. 8: 2095-2096.

Takeuchi, T. and D. J. Prockop. 1969. Incorporation of L-azetidine-2-carboxylic acid and cis-fluro-L-proline into protocollagen and collagen. Biochim. Biophys. Acta 175: 142-155.

_____, J. Rosenbloom, and D. J. Prockop. 1969. Inability of cartilage cells to extrude collagen polypeptides containing L-azetidine-2-carboxylic acid or cis-fluro-L-proline. Biochim. Biophys. Acta 175: 156-164.

Trasko, C. S., C. Branzblau, and R. F. Troxler. 1976. Incorporation of L-azetidine-2-carboxylic acid into hemoglobin S in sickle erythrocytes in vitro. Biochim. Biophys. Acta 447: 425-435.

Troxler, R. F. and A. S. Brown. 1974. Metabolism of L-azetidine-2-carboxylic acid in the alga Cyanidium caldarium. Biochim. Biophys. Acta 366: 341-349.

Youngpeter, J. M. 1964. Spalteholz preparations. Ward's Bulletin 3: 7.

Zameraeva, T. V., A. D. Bibikova and V. I. Mazurov. 1969. Action of azetidine-2-carboxylic on protein synthesis in cartilage tissue of chick embryos. Biochim. Biophys. Acta 34: 136-143.

_____ and V. I. Mazurov. 1970. Action of azetidine-2-carboxylic acid on collagen synthesis in mouse embryos. Biochim. Biophys. Acta 35: 243-252.